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**The Immunocytochemical Localization of Transgenically-expressed
Proteins in Plants**

by

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B.Sc. Hons. (Durham)

A thesis submitted in accordance with the requirements for the degree of
Master of Science of the University of Durham

Botany Department, University of Durham

July 1988

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ABSTRACT

A range of tissue preparation and immunocytochemical localization techniques were assessed for their possible application in the study of the transgenic expression of the *legA* gene, encoding *Pisum sativum* legumin in transformed *Nicotiana plumbaginifolia* seeds.

Immuno-gold localization methods were developed at the EM level, using sections of L.R. White embedded seeds. An endogenous legumin-like protein in the *Nicotiana plumbaginifolia* seeds was recognised by antibodies raised against *Pisum* legumin. In order to determine the temporal and spatial distribution of *legA* encoded legumin in the transformed seeds, it was necessary to develop a suitable means of discriminating between *Pisum* legumin and the endogenous protein. Cross reacting antibodies were precipitated out of a polyclonal antiserum by pre-treatment with an extract from non-transformed *Nicotiana plumbaginifolia* seeds and the remaining antibodies were then used to achieve specific localization of *Pisum* legumin. A method was also developed for the localization of the endogenous legumin-like protein in the non-transformed seeds, using a non-pretreated antibody preparation.

Both proteins were detected in the protein bodies of transformed *Nicotiana plumbaginifolia* seeds, but their temporal and spatial distributions were different. The endogenous protein was abundant in all seed tissues, whenever and wherever there were protein bodies present. *Pisum* legumin was present at a detectable level in the older developing and mature transformed seeds, principally in the cotyledons and adjacent embryo axis and to a lesser extent in the endosperm.

These results clearly demonstrate that the *legA* gene is being differentially expressed, in a manner which is different to the expression of the endogenous legumin-like protein. This suggests that its differential expression is under the control of the non-coding regions of the *legA* gene, rather than being determined by endogenous *Nicotiana* control elements. The significance of these results is discussed in relation to those achieved in other transgenic situations. The usefulness of the methods and their potential future applications are considered.

DECLARATION

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INTRODUCTION

The current high level of interest in plant molecular biology is in part due to the advent of improving techniques for plant genetic manipulation, which open up many new possibilities for academic and commercial exploitation. Genetic engineering of plants can be used as a research tool to gain a greater understanding of the organisation and expression of plant genes and there is also potential for the production of improved varieties, by means which can not only cross the traditional barriers to conventional plant breeding but may also be quicker and more versatile.

There are at present only a few plant species which can be transformed with foreign DNA in a stable manner, such that they are able to pass on the introduced gene to their progeny. Also the number of complete genes which have been isolated, characterised and cloned is limited, though this number is growing rapidly. At the University of Durham Department of Botany, transgenic *Nicotiana plumbaginifolia* plants have been produced which have been shown to express the introduced *legA* gene from *Pisum sativum* L.; with accumulation of the pea seed storage protein, legumin, in *Nicotiana* seeds (Ellis *et al* 1988). The work reported in this thesis concerns the assessment, development and application of immunocytochemical localization techniques to the seeds of such transformed plants in order to determine the details of the temporal and spatial distribution of the pea legumin. For clarity this introduction will be divided into the following sections-

Seed structure and development

Seed storage proteins

Immunocytochemical localization

Plant transformation

Aims of this work



Seed structure and development

In angiosperms a seed is produced by a process of development from a fertilized ovule. One or both of the integuments of the ovule give rise to the testa and the nucellus develops into the perisperm. The two polar nuclei and one male generative nucleus fuse and give rise to the triploid endosperm. The oosphere is fertilized by another male nucleus forming the zygote, which develops into the embryo. The extent of each of these components and the seed structure at maturity varies in different species and many descriptions have been produced, such as the work of Corner (1976) concerning dicotyledon seeds.

Seed development has been investigated and described in many different species; these studies have been reviewed by Dure (1975), Bewley and Black (1985). The extensive studies of the embryogeny, more specifically, have been reviewed by Maheshwari (1950, 1963), Davis (1966), Johri (1984) and Raghavan (1986). In the early stages of seed development the major events are those concerned with cell division and the establishment of the various tissues. Later on the production and deposition of reserves become increasingly the dominant events. The proportions of protein, lipids and carbohydrates and their patterns of distribution throughout the seed varies in different species.

Seed development in pea, *Pisum sativum*, has been investigated and described by a number of workers. Cooper (1938) described the embryogeny from his light microscope observations. Early cell divisions of the zygote produce a globular embryo at the tip of a suspensor and this is surrounded by endosperm. The globular embryo develops with the differentiation of the cotyledons, epicotyl, hypocotyl and radicle and shortly after this the suspensor cells disintegrate. During the course of development there is free nuclear division in the endosperm, with some cell formation in the micropylar portion. Later the endosperm is completely assimilated and the cotyledons then act as the storage organs. Reeve (1948) described the later embryogeny in detail and also described the histogenesis. From their light and electron microscope examination, Bain and Mercer (1966)

described the development of the cotyledons as consisting of four phases: (i) cell division, (ii) cell expansion, (iii) reserve biosynthesis and (iv) seed maturation and dormancy. Further electron microscope examination was performed by Marinos (1970a,b). Hardham (1976) described the pathways of nutrient flow into the developing embryo. In mature *Pisum sativum* seeds the reserves consist of carbohydrates, mostly in the form of starch (52 % dry weight), protein (25 % dry weight) and fat (6 % dry weight); these are stored almost exclusively in the cotyledons which fill most of the volume of the mature seed (Crocker and Barton, 1957).

Seed development in *Nicotiana plumbaginifolia* has been investigated and described by a number of workers. Bhaduri (1936) described the embryogeny of a range of species of the *Solanaceae* including *Nicotiana plumbaginifolia* and found it to be similar to that of *Nicotiana tabacum* as described by Souèges (1920b, 1922). Jos and Sing (1968) described further details especially concerning the endosperm and they conclude that embryo development follows the *Nicotiana* variation of Solanad type (Johansen, 1950). Development of the endosperm starts immediately after fertilization, while division of the zygote is delayed in comparison. The endosperm consists of uninucleate cells from its first cell division throughout development. Once the endosperm has become multicellular the zygote divides to form a linear four-celled proembryo which further develops into a globular embryo at the tip of a suspensor. The globular embryo develops with the differentiation of the cotyledons and embryo axis consisting of radicle, hypocotyl and a small, poorly developed epicotyl. The structure of the mature tobacco seed was described by Avery (1933). The endosperm is retained through to maturity, when it constitutes the greater part of the seed volume. Greenwood and Chrispeels (1985b) have described the cytological details of the mature tobacco seed, from their ultrastructural investigation. The storage parenchyma cells in the embryo and endosperm are similar, containing numerous lipid bodies, a large central nucleus and many protein bodies.

Seed storage protein

Seed storage proteins are accumulated in developing seeds and remain through dormancy to be hydrolysed during germination to provide a supply of amino acids to the seedling during its early development, until it is able to synthesise them for itself. Fulfilling this storage role appears to be their sole function and they are only synthesized and accumulated in seeds, where they are usually in an insoluble and osmotically inactive state, appropriate for seed dehydration. Seed storage proteins have been investigated by many workers and are the subject of numerous reviews, for example Higgins (1984).

Osborne (1924) proposed a system of classifying seed proteins on the basis of their solubility and defined four groups which can be extracted sequentially. Albumins are soluble in water at neutral or slightly acid pHs and are heat coagulatable, many of the proteins in this class are enzymes. Globulins are insoluble in water but can be extracted in salt solutions and are not as readily heat coagulatable as the albumins. Prolamins are soluble in aqueous ethanol but not in water. Glutelins are insoluble in water, salt solutions and ethanol but can be extracted with acidic or alkaline solutions. This classification system is still useful today even though each class contains a heterologous mixture of proteins. The extent to which each of these classes is present in a seed varies according to the species, as does the timing of their synthesis and their distribution. Most of our biochemical and physiological knowledge of seed storage proteins is derived from the species of cereals and legumes which are agriculturally important.

In legumes the seed storage proteins belong predominantly to the globulin group. Osborne divided the globulin of *Pisum sativum*, and later other legumes, into two fractions, vicilin and legumin, on the basis of their precipitation under different conditions. Similar fractions have since been found in a wide range of legumes, and legumin-type proteins have also been found in non-legume species; review by Derbyshire *et al* (1976). Separation of these proteins by ultracentrifugation

generally gives two bands with sedimentation coefficients of approximately 11s for the legumin-type proteins and 7s for the vicilin-type proteins, hence they are often referred to as 11s and 7s proteins. There is some evidence that in many cases the 7s fraction is heterogeneous, consisting of more than one protein. The 11s fraction, however, seems to be more homogeneous. Both the 11s and 7s proteins consist of sub-units, which can be split by electrophoresis, and there is some variety of sub-unit composition of the proteins which vary at different times through seed development. The amino acid compositions of these proteins have been compared in a range of species and generally both the 11s and 7s proteins in different species are of similar composition. These proteins are encoded in multi-gene families: in pea there are 10 genes for legumin and about 15 for vicilin (Boulter *et al* 1987). At the time when the proteins are synthesised these relatively few genes are being expressed at high levels, accounting for about 80 % of seed protein.

In mature legume seeds the storage proteins are found in protein bodies, which are membrane bounded sub cellular vesicles. The protein is synthesised at the rough endoplasmic reticulum (rough ER or RER) by the translation of messenger RNA (mRNA) on ribosomes bound to the ER membrane. From the RER lumen the proteins are transported via the Golgi apparatus, where they may be modified, to the protein bodies (reviewed by Chrispeels, 1985). The vacuoles, which constitute the protein bodies may be derived from the fragmentation, by budding off, of the tonoplast (eg. Craig *et al* 1979, 1980) or by vesicles from the Golgi and/or ER coalescing, or possibly by a combination of these events (eg. Harris and Boulter 1976)

Synthesis and sequestration of seed storage proteins takes place only during the period of deposition of reserves and only in the specific tissues where they are accumulated. In pea, vicilin synthesis and accumulation begins a little before that of legumin, but the latter is accumulated more rapidly and so becomes

more abundant than the former. These genes are expressed predominantly in one tissue, the cotyledons, and hence are of considerable interest for their study as a model system of differential gene expression in plants.

Immunocytochemical localization

The use of antibodies in cytochemical studies to localize specific antigens at the tissue and cellular levels is now a well established technique. A variety of means have been developed to visualize the antibodies in light microscope (LM) or electron microscope (EM) examination. The first to be introduced were the use of fluorescent antibodies (Coons *et al* 1941) for LM examination and the conjugation of ferritin to antibodies (Singer 1959) for EM examination. Conjugation of an enzyme to the antibody and subsequent use of suitable substrate to produce a visible product was introduced by Nakane and Pierce (1966), who used horseradish peroxidase (HRP) and diaminobenzidine (DAB). Ram *et al* (1966) introduced the use of the acid phosphatase enzyme for the same purpose. The use of colloidal gold as a marker for EM examination was introduced by Romano *et al* (1974), antibodies being adsorbed onto its surface.

In order to ensure that the antigen of interest remains in its sites of original occurrence during the localization procedure, the tissue is usually fixed. This introduces the considerable difficulty of preserving the antigenicity of the tissue and often a compromise must be achieved between this and the need to preserve ultrastructural details. Modifications of the standard fixation protocols for conventional ultrastructural studies have included the use of paraformaldehyde-based primary fixatives and the elimination of osmium tetroxide secondary fixation in immunocytochemical studies. Vandenbosch (1986), for example, in a careful, quantitative study of the effects of fixation and embedding resin on immunolabelling of root nodules, found a 5 to 6 fold increase in labelling without osmium tetroxide fixation when using Spurr resin. Such enhancement could, however,

also be achieved by treating ('etching') sections of osmium post-fixed tissue, with periodate prior to immunolabelling. Even higher gains were achieved using hydrophilic rather than hydrophobic resins, and no osmium post fixation, although there is a loss of ultrastructural detail with such treatments.

In order to examine the tissue, sections are usually cut and this may require that the tissue is embedded in a suitable matrix. Immunocytochemical localization can be performed on tissue, as a post- or pre-embedding procedure. For detailed histo- or cyto-chemical analysis it is usually the case that immunolocalization is carried out on sections from frozen or embedded blocks, although in the latter case there is the additional problem of some degree of hindrance to the antibody's movement through the tissue by the embedding matrix.

There have been relatively few accounts of immunolocalization after cryo-microtomy/ultramicrotomy of multicellular plant tissues, although, for example, Baumgartner *et al* (1978) and Greenwood and Chrispeels (1985a) have demonstrated that excellent results are obtainable at optical and electron microscope levels. Because of the technical problems associated with freezing plant tissue without ice damage, many investigators have opted for immunolabelling of sections cut from embedded tissue. The earlier use of the hydrophobic epoxy resins such as Spurr or Epon for EM immunocytochemistry (eg. Craig and Goodchild, 1984) with or without 'etching' which sometimes enhances immunolabelling, is now being replaced by specimen embedding procedures employing the more hydrophilic acrylic resins such as Lowicryl K4M (eg. Roth *et al* 1981; Robertson *et al* 1984), which is usually used for low-temperature embedding and L.R. White which can be used to give sections for both LM and EM immunolabelling studies (eg. Craig and Miller, 1984; Vandenbosch, 1986; Harris and Croy, 1986).

In pre-embedding localization, in which the immunolocalization reactions are performed with the tissue block, there is also limited mobility of the antibody through the tissue. The major disadvantage of pre-embedding localization, how-

ever, is that it tends to be a longer procedure because each localization experiment must be followed by dehydration and embedding. In post-embedding localization the same block of tissue can be used for numerous experiments and controls can also be prepared using adjacent sections, rather than from separately processed tissue samples.

Much of the early botanical work employing immuno-cytochemistry was concerned with distributional studies of seed storage proteins which are present in relatively large amounts. More recently the technique has been applied to the study of less abundant components such as membrane proteins (eg. of oil body membranes- Herman, 1987; chlorophyll a/b binding protein- Høyer-Hansen *et al*, 1988), enzymes (eg. uricase- Vandenbosch and Newcombe, 1986; glyoxysome/peroxisome transitions- Nishimura *et al*, 1986; photorespiratory enzymes- Rawsthorne *et al*; chalcone synthase- Beerhues *et al*, 1988), the cytoskeleton (reviewed by Lloyd, 1987), cell wall proteins (eg. Anderson *et al*, 1987; Stafstrom and Staehelin, 1988) and even plant growth regulators (eg. abscisic acid- Sossoantzov *et al*, 1986; cytokinins- Eberle *et al*, 1987).

Plant transformation

Interest in plant gene expression has increased rapidly in the last few years. This is partly because of the possibility of genetically engineering plants and partly because of the potential for understanding the biology of plants in terms of molecular events. Transgenic plants, which are able to pass on the introduced genes to their progeny, have been produced by a number of workers; the variety of methods has been reviewed by Schell (1987). In order to produce such plants the following components are required- (i) a DNA transfer system, (ii) a selection system to separate transformed material from the original non-transformed material and (iii) a re-generation system to produce mature plants from the transformed material.

Of the various ways of introducing foreign DNA into plant cells that have been used, the interaction of *Agrobacterium* with susceptible plant cells has been the method of choice for many workers. Non-oncogenic vectors derived from the Ti (tumour inducing) plasmid of *Agrobacterium tumefaciens* have been developed (Horsch *et al* 1984) (Zambryski *et al* 1983) which are able to transfer DNA, without causing the disease symptoms typical of infection. A number of different selectable marker and reporter genes, which are introduced alongside the gene of interest between the tDNA borders, have been used to separate transformed material from non-transformed. Selectable markers typically confer some antibiotic resistance to transformed cells so that in the presence of the antibiotic only transformed material will survive. Reporter genes typically encode an enzyme whose activity can be assayed easily in order to show which material is transformed.

Transformation by *Agrobacterium* occurs at the level of individual cells, in order to regenerate a mature plant the transformed cells are taken through a process of tissue culture. Appropriate methods for tissue culture and regeneration of whole plants have been developed for comparatively few plant species as yet. There are well established methods for tobacco and some related species and hence these have been used in many transformation experiments. Suitable methods have not yet been developed for most of the economically important species, where transformation might in the longer term be more useful.

A number of workers have used the transgenic system as a means of investigating the role of the non-coding regions of the genes of interest. By isolating the gene from its normal environment and choosing the extent of the flanking sequences that are transferred it has been possible to determine some of the functions of cis-acting factors. Recent examples of this type of work include Chen *et al* (1988), Voelker *et al* (1987) and Hoffman *et al* (1987).

In pea the *legA* gene is regulated both temporally and in a tissue-specific manner and it is believed that this is partly controlled at the gene transcription level

(Evans *et al.*, 1984; Gatehouse *et al.*, 1982). Comparisons of legumin, legumin-like and other genes have led to the identification of a number of consensus 5' nucleotide sequences, which are obvious candidates for investigation as putative regulatory elements (Baumlein *et al.*, 1986; Gatehouse *et al.*, 1986). In order to investigate legumin gene regulation, a 3.4 kilobase pea genomic DNA fragment, containing the legumin A gene (*legA*) and its intact 5' flanking region, was transferred to *Nicotiana plumbaginifolia* (Ellis *et al.*, 1988).

Cells from *Nicotiana plumbaginifolia* leaf pieces were transformed using an *Agrobacterium tumefaciens* strain containing the Bin 19 binary vector system (Bevan 1984). Transformed plants were regenerated from single transformed cells via tissue culture and from these plants viable (S1) seeds were produced by self fertilization of the initial transformant (Ellis *et al.*, 1988). Enzyme-linked immunosorbent assays (ELISA) were performed on the pooled seed extracts (25 S1 seeds) of *Nicotiana plumbaginifolia* to determine the presence or absence of legumin, and hence the parent plants were characterised as either transformed or non-transformed (Ellis *et al.*, 1988).

With further refinement of the ELISA technique (Kang *et al.*, 1988) it became possible to assay extracts of individual seeds. The characterization of the phenotype of individual seeds allowed the genotype of the parent plant to be deduced. The ratio of legumin expressing to non-expressing seeds, produced as a result of selfing the original transformed plants, was found to be 3 to 1. This ratio could best be explained if the first generation transgenic plants were to be transformed with a single copy of the DNA and hence were hemizygous.

Aims of this work

The work described in this thesis is concerned with an examination of the temporal and tissue specific expression of the *Pisum sativum legA* gene in the progeny

of transformed *Nicotiana plumbaginifolia* (Ellis *et al*, 1988). The temporal and spatial distribution of the encoded protein will reflect the role of the non-coding sequences in the *legA* gene, although there may also be very considerable influences resulting from endogenous trans-acting sequences and post-transcriptional controls.

Immunocytochemical localization was chosen because it is capable of investigating gene expression within host tissues, individual cells and organelles. Western blots and ELISA rely on preparing an extract from a sample of tissue and so the degree of resolution that can be achieved is limited by the minimum sample size that can be used. The results obtained are necessarily an average of the individual contributions of all the cells present, with any non-contributing cells having a diluting effect.

The analysis of *Pisum sativum legA* expression was complicated by the presence of a similar endogenous legumin-like storage protein in non-transformed *Nicotiana plumbaginifolia*, and techniques to discriminate between this and the transgenic product had to be established before the analysis of the transgenic product could be demonstrated. Various means could be tried which may be able to enhance the discrimination between the endogenous and the introduced proteins. One option would be to adjust the physical and chemical environment of the immunocytochemical localization procedure in order to find conditions that favour the recognition of the introduced protein, but not the endogenous protein. Examples of this approach might include the use of a detergent, high salt concentrations, a particular pH, high or low temperatures, long or short incubation times, dilution, different washing procedures and various combinations of these factors.

Another option would be to investigate a variety of different antibodies and the ways in which they are prepared and purified which may also be important in determining the degree of discrimination that can be achieved. A monoclonal antibody might be prepared which would only recognise a single antigenic deter-

minant found only on the introduced protein. A polyclonal antibody preparation may be affinity purified or immuno-precipitated with a suitable protein extract in order to remove any cross reacting material. The relative contributions that these factors might make to enhancing the discrimination between the introduced and endogenous proteins, needed to be assessed in order to develop an appropriate immunocytochemical localization technique.

MATERIALS

Plant Material

Seeds of *Nicotiana plumbaginifolia* were obtained as a dihaploid stock from the Institut National de la Recherche Agronomique, Versailles, France. Plants were grown in Levington's Universal compost (Fisons, Ipswich, U.K) in a controlled environment of 25 °C, 70 % relative humidity, and 18 hour photoperiod (200 $\mu\text{mol}/\text{m}^2/\text{s}$ PAR). Seeds were collected from these plants and from similarly grown transgenic plants (Ellis *et al*, 1988).

Antisera

Anti-legumin polyclonal antisera were raised by injection of purified legumin in rabbits. The legumin had been purified from pea seed extracts by ammonium sulphate fractionation and hydroxylapatite chromatography (Gatehouse *et al*, 1980). Legumin specific antisera were obtained by purification using immunoaffinity chromatography (Croy *et al*, 1980). These anti-legumin sera, used in this work, were provided by Drs. Gatehouse and Croy of University of Durham, Department of Botany.

Various crude rabbit and mouse anti-legumin polyclonal antisera and monoclonal mouse anti-legumin antibodies, were provided by Dr. Kang of University of Durham, Department of Botany.

Chemicals

Unless otherwise stated, all chemicals used were of analytical grade and were obtained from either-

BDH, Poole, Dorset, UK. or SIGMA CHEMICALS, Poole, Dorset, UK.

Electron microscopy materials

Spurr resin, reagents and consumables for ultrastructural studies were obtained from TAAB Laboratories, Reading, Berkshire, UK.

L.R. White resin was obtained from Agar Aids, Stansted, Essex, UK.

Secondary antibodies which were gold-labelled or enzyme-linked and Protein A-gold were obtained from Janssen Pharmaceutical Ltd., Wantage, Oxon, UK. or SIGMA.

Silver enhancement reagents (IntenSE II kit) were obtained from Janssen.

METHODS

Preparation of material for microscope examination

Fixation- Seed capsules of a range of stages of development were harvested from both transformed and non-transformed *Nicotiana plumbaginifolia* plants. The capsules were cut open with a razor blade and thoroughly shaken in fixative solution to remove the seeds as they were harvested. The seeds were then nicked with a razor blade under a stereo microscope, within an hour of harvesting (either still in fixative or briefly transferred to water and then back to fixative). This treatment was found to improve the penetration of fixative into the seeds past the seed coat and nucellus, which if left intact impede infiltration of the seeds.

Mature seeds were collected when their capsules had dehisced, and the seeds were stored, dry at room temperature, until needed. They were then nicked and put into fixative.

Two fixatives were used, a standard one (ST), intended to give good conventional ultra-structural preservation, and an immunocytochemical one (IMM), intended to give an acceptable preservation but with minimal damage to antigenicity or obstruction of antigenic sites. ST consisted of 1.5 % formaldehyde (made from paraformaldehyde) and 2.5 % gluteraldehyde in 0.05 M sodium cacodylate, buffered at pH 7.0, and IMM consisted of 2.5 % formaldehyde and 1.5 % gluteraldehyde in 0.05 M sodium phosphate buffer at pH 7.0. Seeds were fixed at 4 °C for 16 hours. Excess un-incorporated fixative was removed by washing twice for 30 minutes, with the same buffer as in the fixative. The ST fixed seeds, only, were post-fixed with 1 % w/v aqueous osmium tetroxide at 4 °C for 16 hours.

Dehydration- In preparation for infiltration with hydrophobic embedding me-

dia, the seeds were dehydrated by taking them through a graded series of aqueous ethanol. Tissue was incubated in 12.5 %, 25 %, 50 %, 75 %, 95 % ethanol each in turn (2 times 30 minutes) and then 100 % (dried over molecular sieve) ethanol (4 times 30 minutes).

Embedding- The ST fixed seeds were embedded in Spurr resin (Spurr 1969) and the IMM fixed seeds in L.R. White resin (London Resin Company). The seeds were infiltrated, on a rotator, with a 1:1 ethanol:resin mixture over-night (16 hours) at room temperature; this was then followed by infiltration with pure resin for 32 hours (resin was changed after 8 hours, 16 hours and 8 hours, to prevent its thickening). The seeds and resin were then transferred to capsules (air-tight and filled to the brim, for L.R. White resin), labelled and solidified by polymerisation at 70 °C for 16 hours.

Seeds were also embedded in either polyethylene glycol (PEG 1500) or wax after both ST and IMM fixation and dehydration. They were infiltrated with PEG or wax, at 56 °C, starting with a 1:1 mixture with ethanol, followed by changes of fresh PEG or wax at the same time intervals as for resin. The seeds were then transferred to capsules and the PEG or wax was solidified by cooling to 4 °C.

Block trimming- The capsules were cut open with a razor blade to release the block of hardened resin, wax or PEG containing the embedded seeds. Blocks were held in clamps, aligned with the required seeds accessible, and excess resin, wax or PEG, over and around the seeds, was pared away with razor blades, to give a pyramid with a rectangular flat topped block-face at the apex. The block-face was typically trimmed to 0.5-3 square mm if it was intended to cut sections for EM examination and up to 40 square mm for LM.

Cutting sections- Sections of resin embedded material were cut, using an LKB ultratome (type 4801A, Stockholm, Sweden), with glass knives, fitted with a water filled reservoir behind the cutting edge. As they were cut, the sections floated on the water surface, where they were spread out using a heat-pen to remove the wrinkling caused by sectioning (IMM sections were subjected to the minimum possible heat-pen exposure to avoid any unnecessary risk of heat damage to antigenic sites). Sections for LM examination were cut to give an approximate thickness of 1 μm , and then transferred, in a drop of water in a wire loop, onto a glass slide. Sections were dried down onto the glass over a hot-plate (kept as cool as possible for IMM sections); sections dried down by evaporation, without the use of the hot plate were found not to adhere well. Sections for EM examination were cut to give an approximate thickness of 50-100 nm (silver or gold in colour) and then picked up on formvar-coated Ni or Cu/Rh 200 mesh grids.

Sections of PEG and wax embedded material were cut, using a Leitz 1512 microtome, for LM examination at a thickness of approximately 10 μm . Sections were transferred onto the surface of water in a trough to spread out and then onto glass slides, as above; PEG was dissolved out of the sections whilst they floated on the water.

Staining and mounting for LM examination- The sections were stained by covering them with a pool of 1 % w/v toluidine blue (made up in a 1 % w/v sodium tetraborate solution). Sections with stain were warmed on a hot plate with the staining time (typically for 30 seconds) determined by watching the colour develop to the desired level and then washing away the excess stain with water. The sections were dried over the hot plate and examined under the microscope before they were either (i) left un-mounted, (ii) temporarily mounted in water or Histomount or alternatively (iii) permanently mounted in the same resin as that in which they were embedded and covered with a cover slip.

Staining for EM examination- The sections were stained by floating the grids on a drop of saturated aqueous, or (to enhance the contrast) 70 % ethanolic, uranyl acetate solution for 15 minutes at room temperature. They were washed with a jet of distilled water and blotted dry. The sections were usually then further stained with Reynold's lead citrate (Reynolds, 1963) for 15 minutes at room temperature, taking care to minimise the stain's exposure to carbon dioxide.

Examination- A Leitz Ortholux microscope, fitted with a Leitz epi-fluorescence illuminator attachment, and a Nikon Diaphot TMD inverted microscope, fitted with a Nikon Diascopic DIC Nomarski attachment and a Nikon Epi-fluorescence TMD-EF attachment were used for LM examination, with or without their attachments as required. A Philips EM 400 transmission electron microscope was used for EM examination.

Photography- Photographs were taken using Orthomat camera equipment for the Leitz microscope and a 35 mm Nikon FE camera, front mounted, for the Nikon microscope. Ilford XP1 400 ASA or Kodak Technical Pan 50 ASA were used for black and white prints; Kodacolour 400 ASA for colour prints and Fujichrome 100 ASA for colour slides. For the EM, the plate camera was used with Kodak 4489 electron microscope film. Black and white prints were made on Ilford Ilfospeed multigrade II photographic paper, using appropriate contrast filters.

Immunocytochemical localization

The general procedure that was used for immunocytochemical localization consisted of a blocking step, a primary antibody incubation, a secondary antibody incubation and a visualization step, with washes between each, and then the

material was examined and the labelling assessed. The blocking step was intended to saturate the capacity of any substances in the material which could non-specifically bind antibodies, with a suitable protein (eg. a 1 % w/v solution of bovine serum albumin, BSA). The primary incubation was with an antiserum raised specifically against the protein of interest (eg. *Pisum* legumin). The secondary incubation was with a labelled or modified antiserum raised against serum of the species in which the primary antibodies were raised. The secondary antibody was labelled (eg. adsorbed onto gold colloid particles) or modified (eg. conjugated to an enzyme) so that it could be seen after a suitable visualization step (eg. silver enhancement or incubation with a substrate for the enzyme). A buffer (eg. phosphate buffered saline, PBS or 2-amino-2-(hydroxymethyl)-1,3-propanediol buffered saline, Tris/NaCl) was used for washes and to dilute the blocking protein and antisera for the incubations. Buffers were used with or without a detergent added (eg. PBS containing 0.1 % Tween-20, PBST) and/or a blocking protein (eg. PBST containing 0.1 % BSA, PBSTB). Examination was either by light or electron microscope and the labelling was assessed in comparison with results from suitable control experiments.

Post-embedding localization at LM level- Transformed and non-transformed *Nicotiana plumbaginifolia* seeds were fixed (IMM), dehydrated, embedded in L.R. White resin. Sections were cut at a thickness of 1 μ m and dried down onto glass slides. The sequential incubations and washes were all performed by covering the sections with a pool (of 5 μ l to 200 μ l) of each reagent (in turn on the slide). To prevent evaporation during the incubations, the slides were kept in a humid chamber (a closed petri-dish with wax supports to hold the slide over a wet filter paper). Reagents were removed by tipping them off the slide and then wiping around the sections with paper towel, the next reagent was then added immediately (before the sections could dry out completely).

Localization was attempted with anti-legumin primary antiserum, using three different secondary antibody and visualization systems-

(i) A secondary antibody conjugated to a peroxidase enzyme, visualized by the production of a coloured insoluble product from a colourless, soluble substrate. Two different substrates were used, one was- 3,3'-diaminobenzidine tetrahydrochloride (DAB), which was freshly prepared by dissolving 1 mg in 2 ml of PBS containing 2 μ l of 30 % hydrogen peroxide and filtering; it was used either with or without the addition of 60 μ l of 1 % aqueous cobalt chloride, which if present enhances the colour from brown to black. The other substrate used was 4-chloro-1-naphthol (4CN), which was prepared by dissolving 1 mg in 10 μ l of ethanol, adding this to 2 ml of PBS containing 2 μ l of 30% hydrogen peroxide and then filtering.

(ii) A secondary antibody conjugated to rhodamine, a fluorescent dye, visualized using a 546 nm excitation filter and a 580 nm dichroic mirror and barrier filter.

(iii) A secondary antibody adsorbed onto the surface of colloidal gold particles, with mean diameters of 5 nm to 20 nm, visualized by silver enhancement of the gold particles, which would otherwise be too small for conventional optical microscopy, with a metallic silver precipitate.

Post-embedding localization at EM level- The same IMM fixed and L.R. White embedded seeds were used as for the LM procedure. Sections were cut at a thickness of approximately 100 nm and recovered on formvar-coated EM grids (Cu/Rh 200 mesh). Incubations and washes were all performed by floating the grid on a drop (of 3 μ l to 10 μ l) of the reagent, resting on a piece of Nescofilm in a humid chamber. Reagents were removed by lightly blotting the grid with filter paper and then immediately placing the grid on the next reagent.

Anti-legumin primary antibodies were used, with secondary antibodies adsorbed onto colloidal gold particles of a range of sizes (5 nm to 20 nm), which are large enough to be seen directly by EM examination. Sections labelled with 5 nm gold particles were observed un-stained or stained with uranyl acetate only, for easier recognition of the label; other sections were stained with both uranyl acetate and lead citrate.

Silver enhancement at EM level- Sections were recovered on formvar-coated nickel 200 mesh EM grids. Incubations were performed as detailed above with anti-legumin primary antibodies and colloidal gold-labelled secondary antibodies. After thorough washing in buffer, followed by distilled water the labelling was silver enhanced by floating the grids on drops of reagent. After washing with distilled water, staining was performed with both uranyl acetate and lead citrate.

Pre-embedding localization- Localization was also attempted in seed material which had not been embedded, and also in material which had not been either fixed or embedded. The procedure was similar to that for post-embedding localization. Seeds were either immobilized by adhering to the surface of glass slides with dental wax or embedding wax or free floating in each reagent. Immobilized seeds were cut in half with a razor blade, with the cut surface facing upwards and approximately level with the surrounding wax. Free seeds were cut in half and immediately transferred to a millipore Swinnex-13 filter unit, containing either buffer or fixative. Incubations and washes of the free seeds were all performed by pushing the reagents through the filter unit with a syringe. The rest of the immunolocalization steps were then performed as for post-embedding LM or EM localization, as appropriate. After the visualization step the seeds were examined under the LM with the use of illumination from above. The seeds were then fixed

(IMM or ST, but without osmium tetroxide), embedded in resin and sectioned for LM or EM examination, or both.

Visualization for both LM and EM examination of the same seed was attempted either by an incubation with protein A-gold as well as with an enzyme-linked secondary antibody, or by silver enhancement of a gold-labelled secondary antibody.

Development of reliable immunocytochemical localization techniques

(i) **Physical and chemical factors-** The localization procedures described above were performed under a variety of physical and chemical conditions in order to assess the influence of these factors on the labelling. These included the use of a range of different temperatures, incubation times, dilutions of reagents, washing protocols, buffers, pH, salt and detergent concentrations, blocking proteins and primary antibodies. These were investigated both in isolation and in various combinations.

(ii) **The use of seeds of known phenotype-** Two mature seeds from a *LegA* transformed *Nicotiana plumbaginifolia* plant were identified as high legumin expressors, by ELISA of one half of each (Kang, personal communication). The corresponding other halves were IMM fixed, embedded in L.R. White resin and sections were cut and used for immunocytochemical localization at both LM and EM levels as described above.

(iii) **Identification of a homozygous legumin positive line, by ELISA-** Individual seeds were separately ground up in 200 μ l of extraction buffer (0.2 M Tris/HCl, 0.3 M NaCl, pH 9.5) to liberate their constituent proteins, including any legumin, into solution. The extracts were centrifuged in a bench centrifuge

for 1 minute to sediment any solids and 50 μ l of the aqueous phase was loaded into microtitration plate wells, one extract per well. Proteins were then absorbed onto the surface of the wells by a 1 hour incubation at 37 °C, after which the extraction solution was removed. Remaining traces of un-absorbed or loosely bound proteins and any other extract components were removed by washing the wells with PBST at pH 7.5. A 1 % BSA solution was then incubated in the wells for 30 minutes at 37 °C in order to saturate the capacity of any proteins which could non-specifically bind antibodies. The BSA was removed and replaced with an anti-legumin primary antibody solution for an incubation of 1 hour at 37 °C. The primary antibody was then removed and the wells were washed with PBST. A peroxidase-secondary antibody complex was then incubated in the wells for 2 hours at 37 °C. The complex was removed and the wells thoroughly washed with PBST. 1 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (ABTS), a substrate for peroxidase which gives a coloured product, was incubated in the wells for 1 hour at room temperature. The relative amounts of legumin originally present in each seed were then deduced from the absorbance due to the product formed in each well, measured at 414 nm by a Titertek Multiskan MCC plate reader.

(iv) Mono-clonal primary antibody- A range of monoclonal mouse antibodies were used as the primary antibody in the localization procedures as described above, using similar IMM fixed and L.R. White embedded seeds.

(v) Pretreatment of the primary antibody- 0.5 ml of primary antibody was diluted to its final working strength in PBSTB. Approximately 50 mature non-transformed seeds were pre-soaked in PBSTB and then crushed between 2 glass microscope slides in 20 μ l of diluted antibody. This was added to the remainder

of the antibody and incubated with rotation for 1 hour at room temperature. The solution was then centrifuged at 12,000 g for 10 minutes and the precipitate discarded. The supernatant was then either used for immunocytochemical localization or was treated again a number of times in the same way.

SDS-polyacrylamide gel electrophoresis- Protein extracts were prepared from pea seeds and from non-transformed *Nicotiana plumbaginifolia* seeds by homogenization in sample buffer (0.2 M Tris/HCl (pH 6.8), 2 % sodium dodecylsulphate (SDS), 10 % sucrose), to give a final concentration of 20 mg/ml of extract. Purified pea legumin was also diluted in the same sample buffer and each of the samples was prepared both with and without the addition of 28 mg/ml of 2-mercaptoethanol. 40 μ l aliquots of each of the samples was loaded per track of a SDS-polyacrylamide gel and electrophoresis was performed at 30 mA for 5 hours.

Western Blotting- Protein bands in the gel were electroblotted onto a nitrocellulose filter (BA85; Schleicher and Schuell; Anderman, Kingston-upon-Thames, Surrey, UK) as described by Towbin *et al* (1979). The filter was then incubated, with shaking, for 30 minutes in a 5 % w/v solution of low-fat dried skimmed milk (Marvel; Cadbury's, Bournville, Birmingham, UK) in 20 mM Tris/HCl (pH 7.2), 0.9 % w/v NaCl in order to block non-specific binding sites. The blot was then screened with pretreated anti-legumin primary antiserum. Immunoreactive bands were visualized with secondary antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, Watford, UK) using DAB as the substrate. The filter was then photographed before being re-screened with non-pretreated primary antibody at the same concentration as the pretreated already used. After repeating the visualization procedure, the filter was re-photographed.

RESULTS

Selection of suitable materials and preparation techniques

As the flowers of *Nicotiana plumbaginifolia* set seed sequentially up the stem, it was possible to harvest seeds at a range of stages of development from the same plant. Mature (M) seeds were sampled from recently dehissed capsules, whilst each capsule above this on the same part of the stem contained progressively younger seeds. There was little variation in developmental age amongst the seeds in each capsule and different plants had seeds of a similar age in capsules in equivalent positions. The fourth capsule above the recently dehissed capsule (ie the M minus 4 or M-4 capsule) contained M-4 seeds with the youngest observed embryos, which consisted of only a few cells. Both the standard (ST) and immunocytochemical (IMM) fixation and embedding procedures described above were found to give good preservation of tissue distribution and cellular details at both the LM and EM levels for both mature seeds and for the four sub-mature stages (M-1 to M-4) from the capsules above. Resin embedded material could be sectioned for both LM and EM levels, but PEG and wax embedded material could only be sectioned for LM level. The quality of the fixation of older seeds was improved by cutting away one end of the seed before fixation to allow better penetration of the reagents into the tissues.

Morphology and cytology of developing *Nicotiana plumbaginifolia* seed

Plates 1 to 4 show the development of the seeds from an early stage to maturity. Plate 1 shows a typical seed from the M-4 capsule, and Plates 2 to 4 show a range of older seeds up to full maturity. Seeds in the M-1 capsule were found to be similar in structure to the mature seeds, though not yet fully dehydrated. Cytological information was gained from LM and EM investigations of the differ-

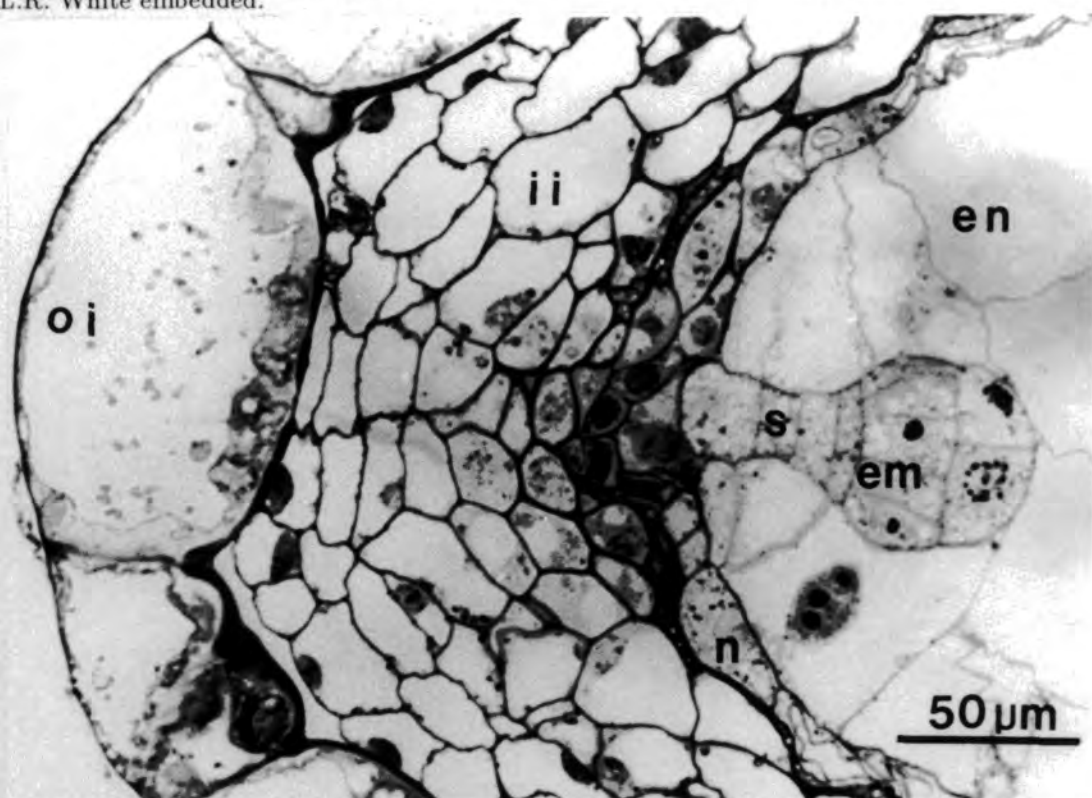
ential staining of cell components. Toluidene blue staining was especially useful to distinguish between protein and lipid at the LM level. At the EM level, cellular components were identified by their appearance in ST fixed and embedded material after staining with uranyl acetate and lead citrate, and where necessary were compared with un-stained or IMM fixed and embedded sections, stained or un-stained.

At the early, M-4, stage of seed development shown in Plate 1, the embryo consists of only a few cells which form a ball attached to the tip of a suspensor, which is projecting into the endosperm. The endosperm is surrounded, in turn, by the nucellus, inner integument and outer integument with its thickened inner cell walls. The embryo cells have large nuclei of varying appearance according to their positions in the cycle of division. The cytoplasm is dense, with prominent mitochondria and only a few small vacuoles and lipid droplets, there are no visible reserves of protein. Thus it appears that the major event at this stage of embryo development is cell division. The endosperm cells are much bigger than those of the embryo, and their volume consists mainly of large vacuoles. The cytoplasm is less dense and the nuclei are not as large and appear to be dividing less frequently.

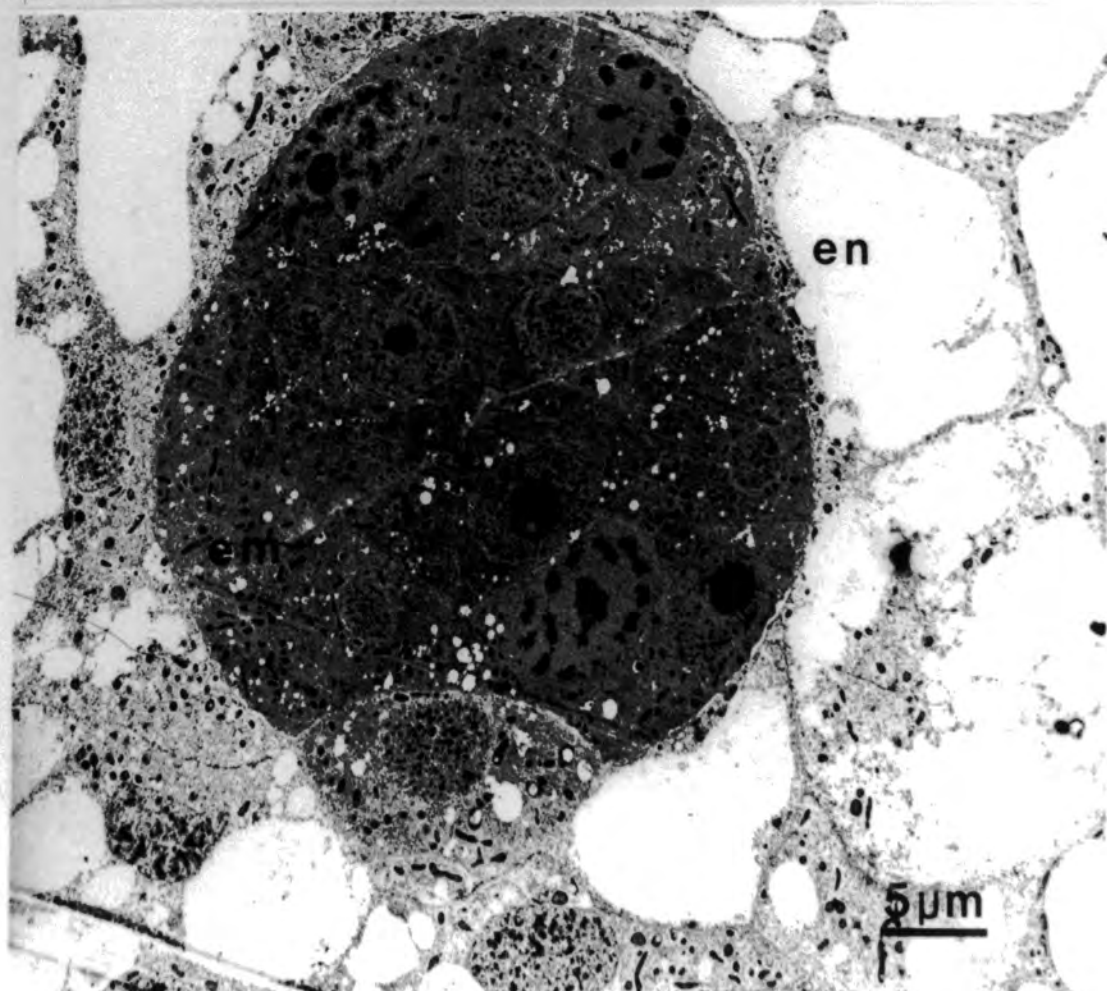
The early phase of cell division gives rise to a larger globular embryo, at the M-3 stage, consisting of an increased number of cells, which appear to be of similar size and ultrastructural details (Plate 2) to the embryo cells at M-4. The endosperm has also increased in size, but now consists of smaller cells, with smaller but more numerous vacuoles and increased lipid reserves, which are in membrane bounded lipid bodies. Small aggregates of protein are now visible in some of the vacuoles. The seed tissues of maternal origin have been pushed outwards by the expanding embryo and endosperm, to give a narrow band around the outside, the outer integument cells are developing further thickening of their inner cell walls.

The embryo grows by continued cell division into a torpedo shape with the cotyledons at one end, at the M-2 stage, shown in Plate 3. The embryo cells contain

Plate 1. *Nicotiana plumbaginifolia* seed at an early stage of development, M-4, IMM fixed and L.R. White embedded.

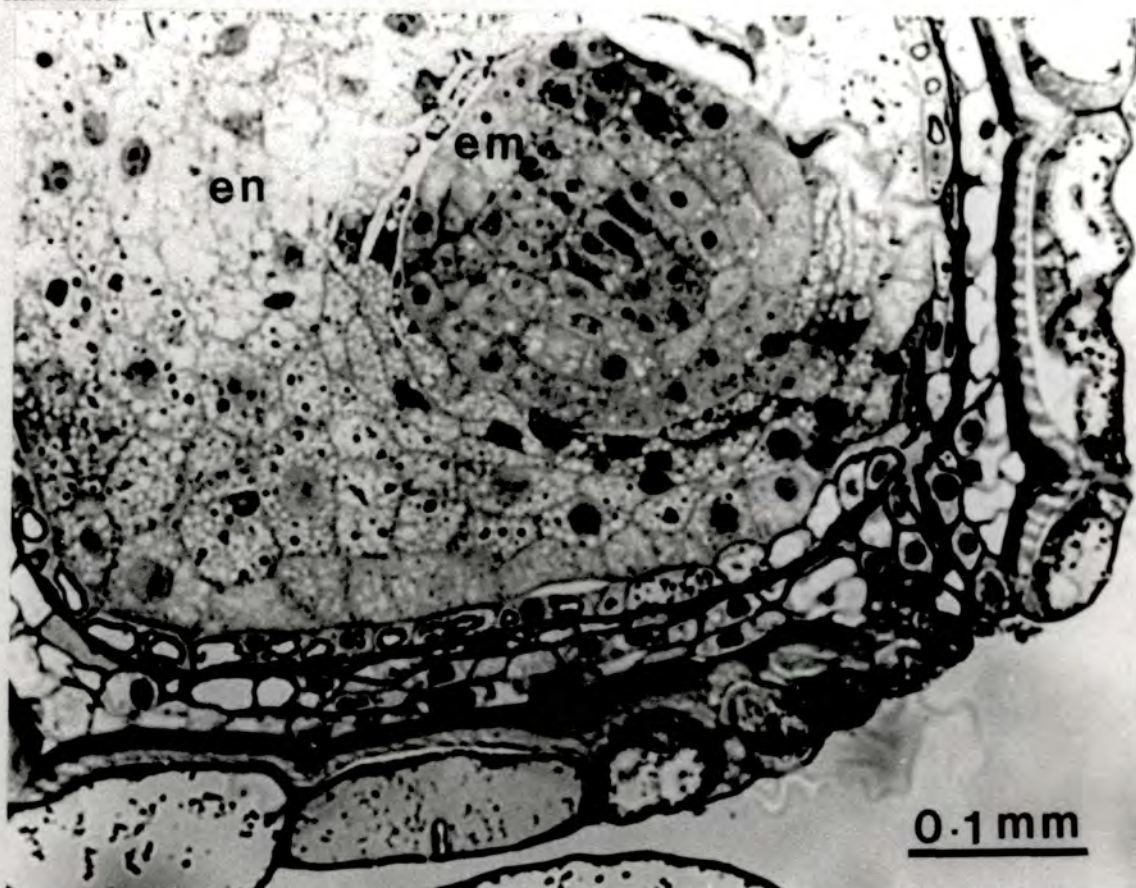


A. Light micrograph of a toluidene blue stained LS. Embryo (em), suspensor (s), endosperm (en), nucellus (n), inner-integument (ii), outer-integument (oi).

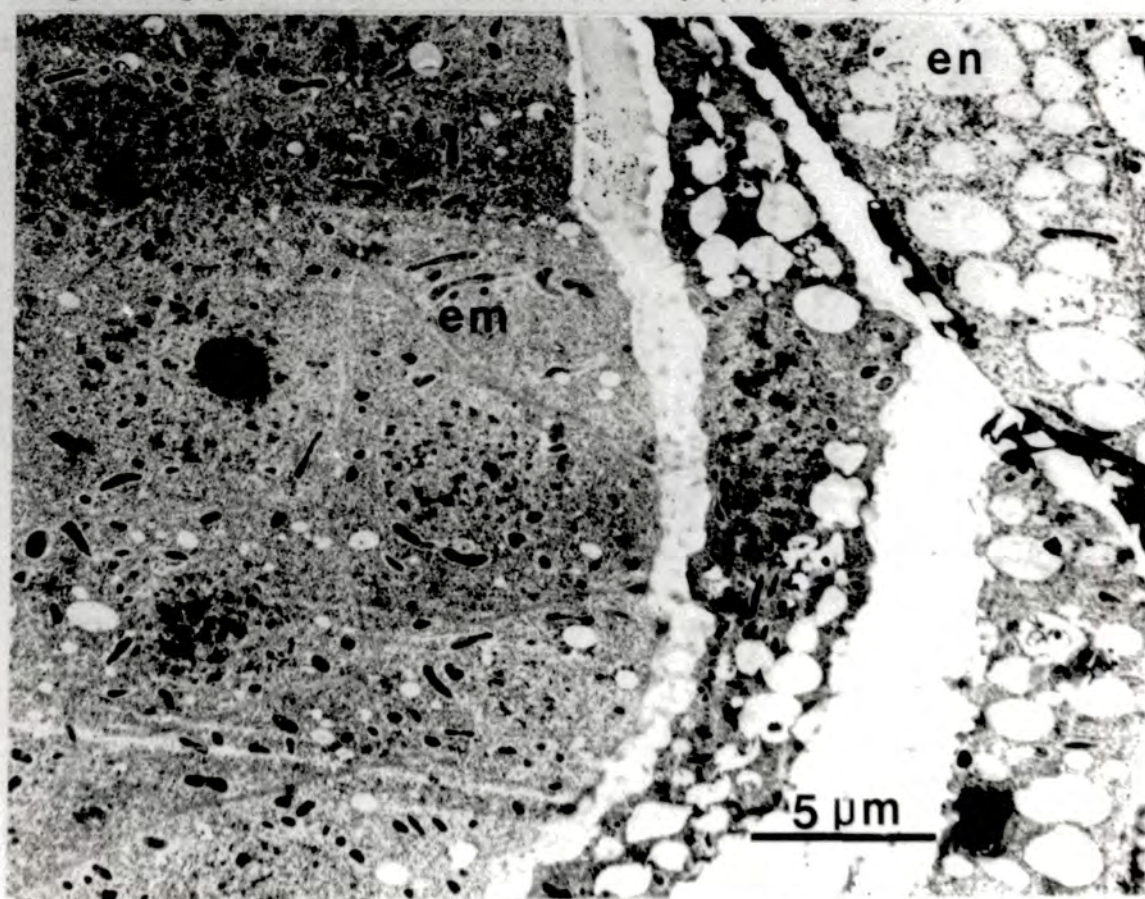


B. Electron micrograph of a uranyl acetate and lead citrate stained LS. Embryo (em), endosperm (en).

Plate 2. Developing *Nicotiana plumbaginifolia* seed at the M-3 stage, IMM fixed and L.R. White embedded.

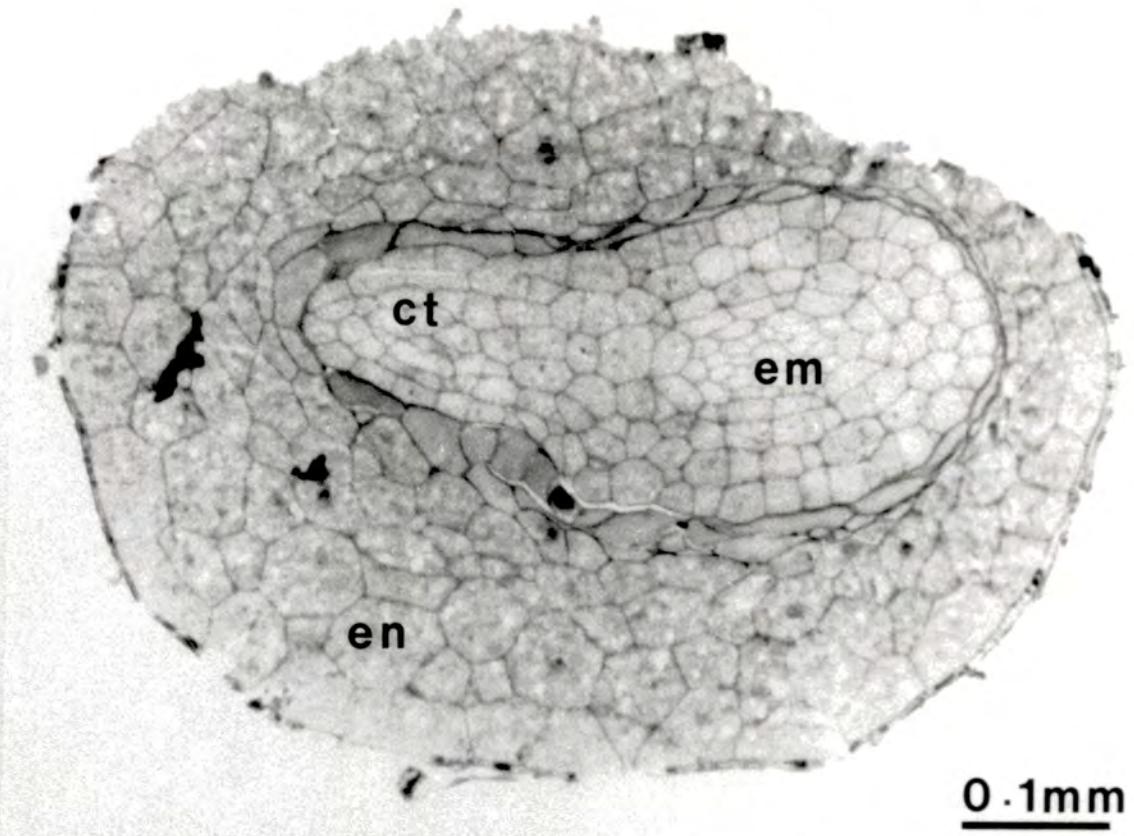


A. Light micrograph of a toluidene blue stained LS. Embryo (em), endosperm (en).

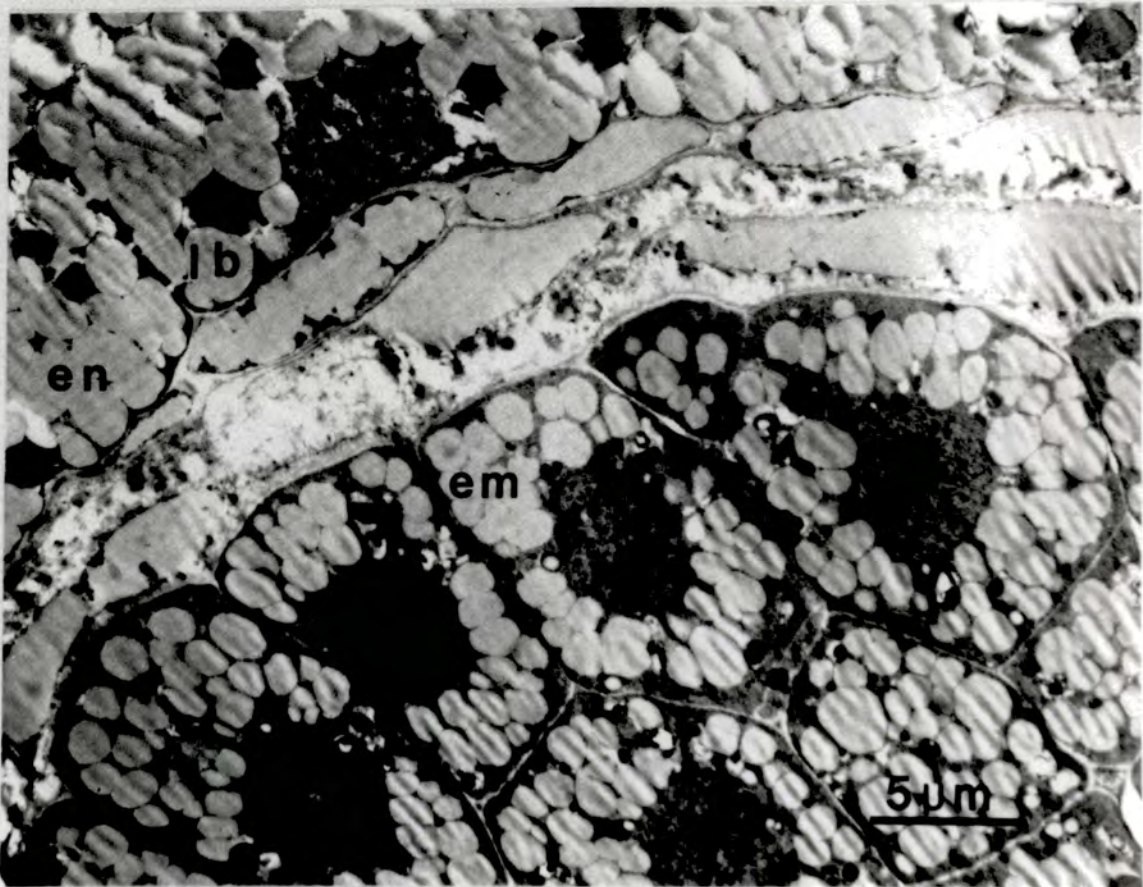


B. Electron micrograph of a uranyl acetate and lead citrate stained LS. Embryo (em), endosperm (en).

Plate 3. Developing *Nicotiana plumbaginifolia* seed at the M-2 stage, ST fixed and Spurr embedded.



A. Light micrograph of a toluidene blue stained LS. Embryo (em), endosperm (en), cotyledon (ct).

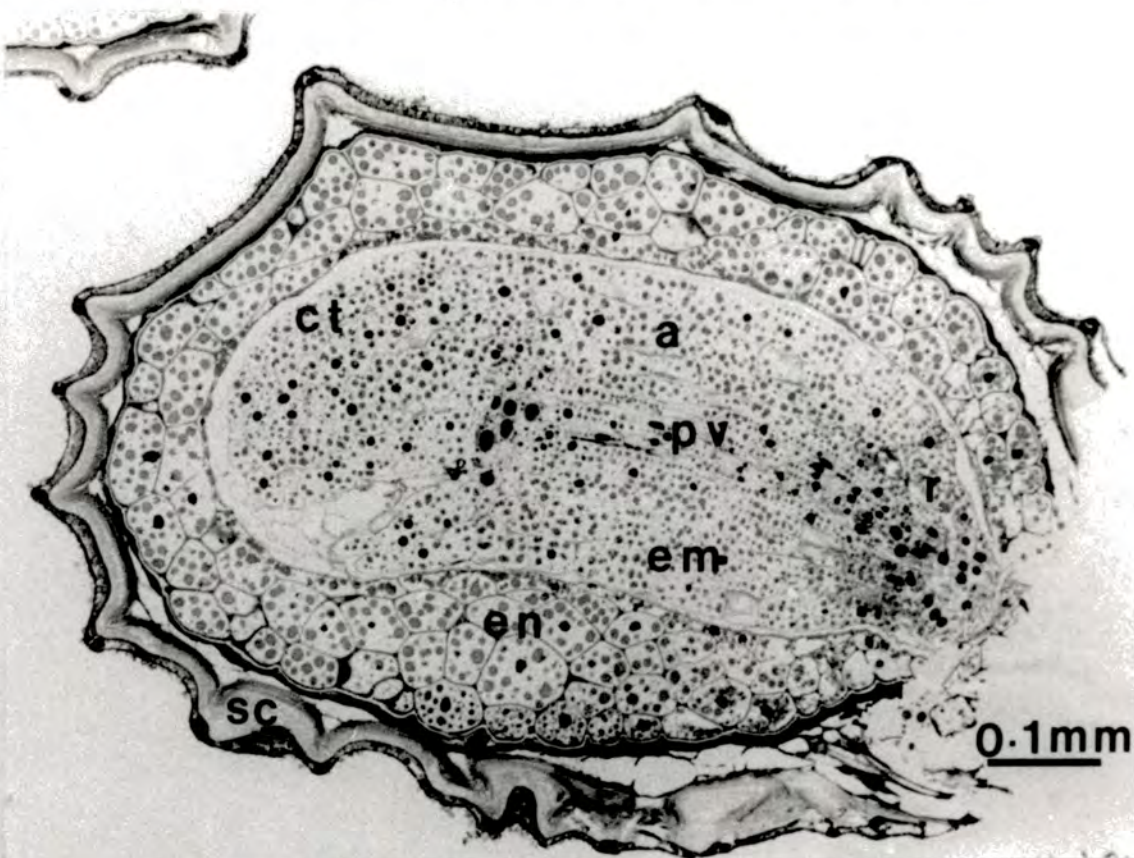


B. Electron micrograph of a uranyl acetate and lead citrate stained LS. Embryo (em), endosperm (en), lipid body (lb), protein body (pb).

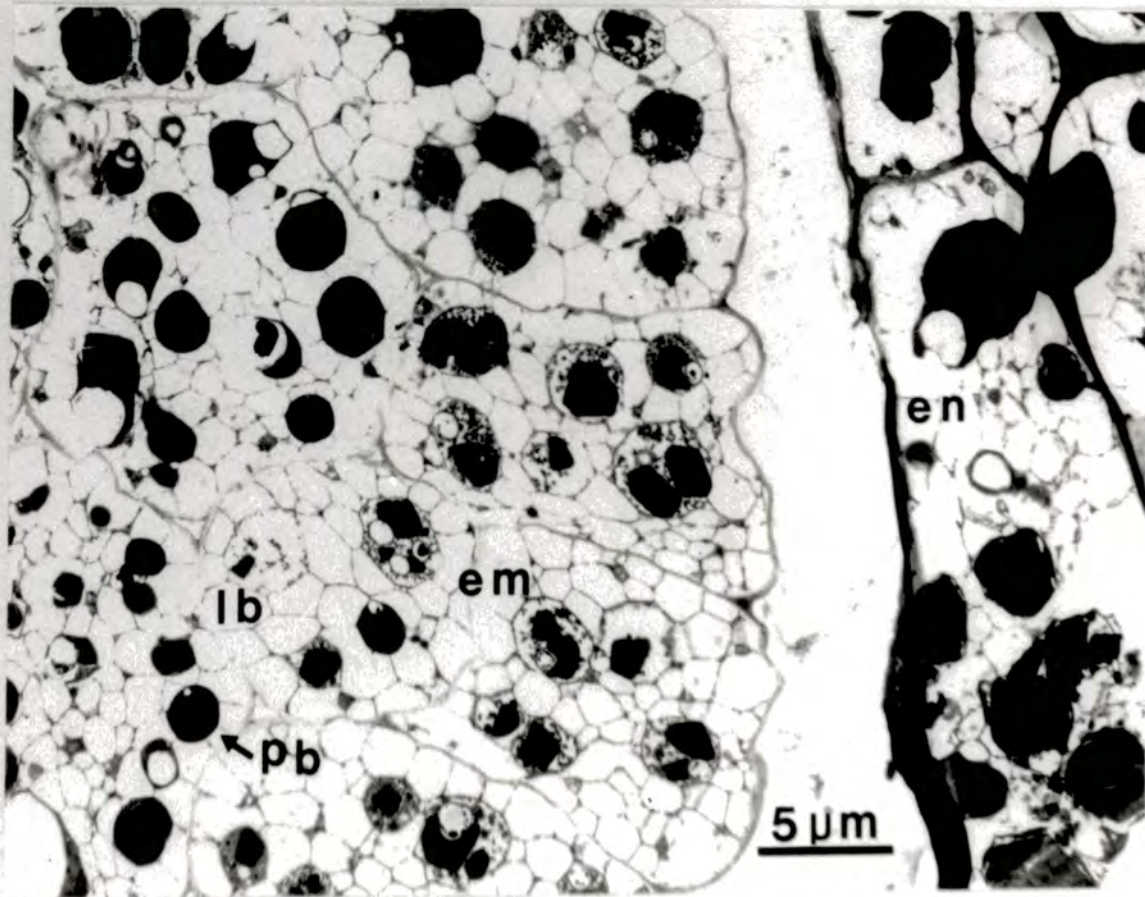
significant numbers of lipid bodies and there is the first appearance of protein reserves. The endosperm morphology has remained similar and the cells are unchanged in their size and shape. The endosperm cell volume is now mainly filled with lipid bodies and to a lesser extent, protein bodies, thus the synthesis and storage of reserves have clearly been the major events. The nucellus and inner integument are greatly reduced in their extent, being crushed between the endosperm and outer integument.

Cell division continues in the embryo with some limited differentiation of proto-vascular tissue and finally dehydration occurs as the seed reaches maturity (Plate 4). Most embryo cells now contain extensive reserves of lipid and protein, but there are less reserves in the elongated cells forming the proto-vascular system through the axis, and the meristem cells in the radicle and hypocotyl contain no visible protein reserves. The endosperm has been pushed outwards, by the growth of the embryo and no trace remains of the nucellus and inner integument. The outer-integument has lost its outer cell walls so that only the thickened inner cell walls remain to form the testa.

The development of the endosperm protein reserves as observed in IMM fixed seeds can be seen in greater detail in Plate 5 and that of the embryo in Plate 6. Intracellular deposition of protein appears to be similar in both tissues, though it occurs earlier in the endosperm. The first protein to be visible is in the form of darkly staining aggregates within vacuoles, the numbers of aggregates, their size and morphology is variable. In ST fixed sections of young seeds the lipid droplets stained darkly enough that they appeared to be protein aggregates free in the cytoplasm, but comparison with IMM fixed sections of similarly aged seeds showed that they are in fact lipid droplets. In the IMM fixed sections it was not possible to distinguish lipid bodies from vacuoles, but comparison with ST fixed sections clearly showed the difference, as can be seen in Plate 7. As more protein is deposited many of the vacuoles became completely filled with protein, making

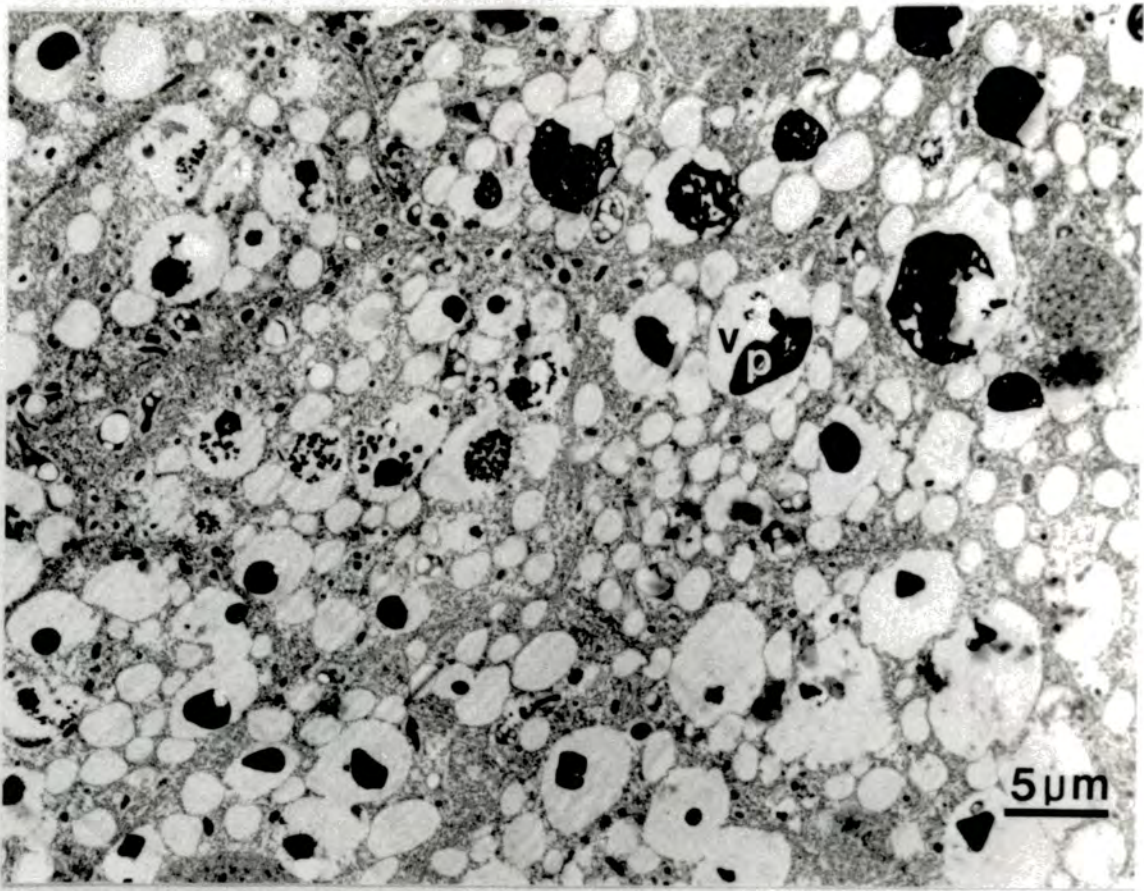


A. Light micrograph of a toluidene blue stained LS. Embryo (em), endosperm (en), cotyledon (ct), proto-vascular tissue (pv), axis (a), radicle (r), seed coat (sc).

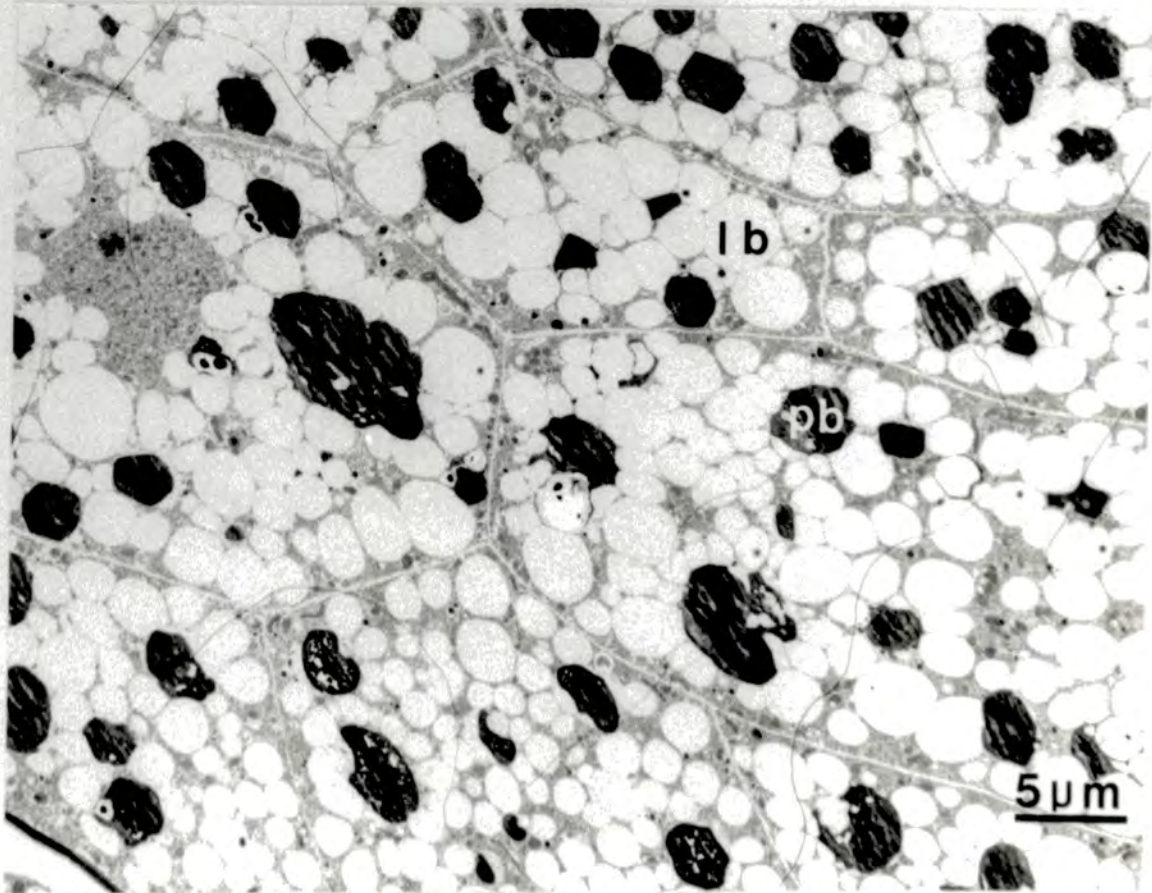


B. Electron micrograph of a uranyl acetate and lead citrate stained LS. Embryo (em), endosperm (en), lipid body (lb), protein body (pb).

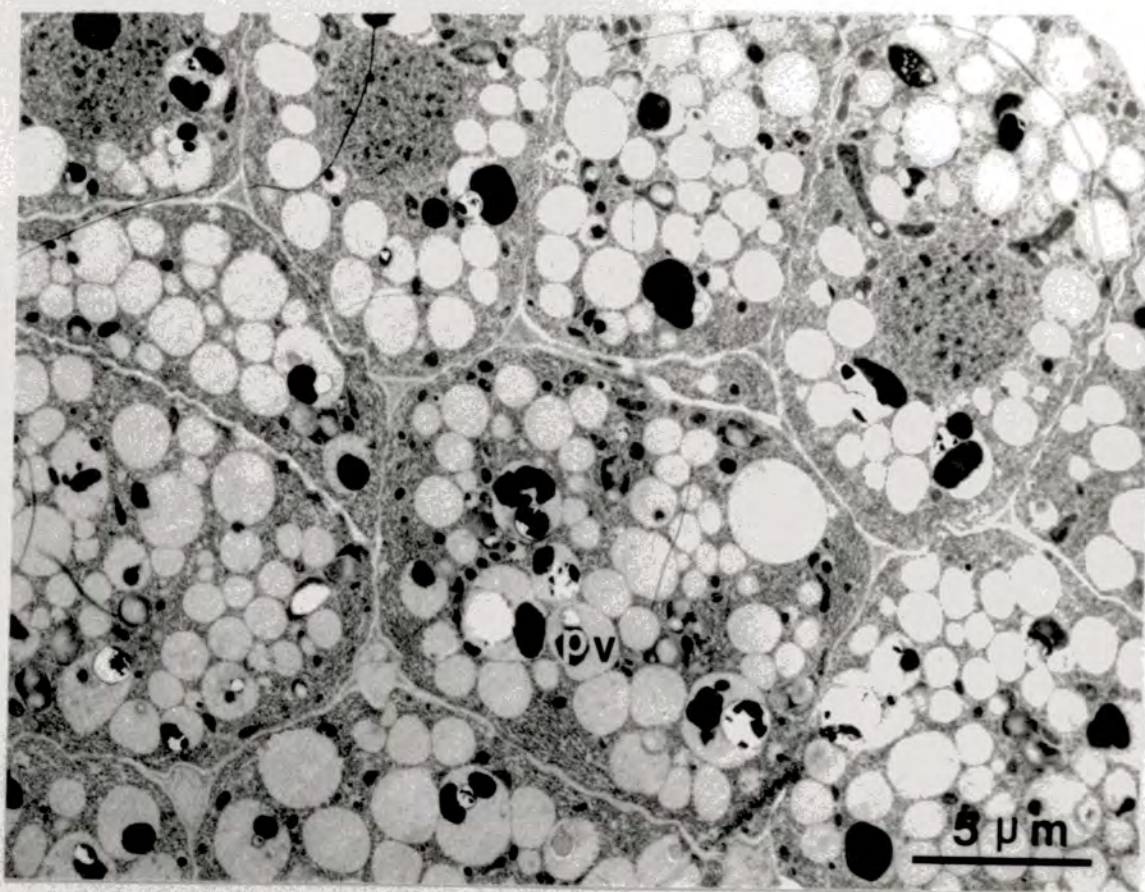
Plate 5. Electron micrographs of IMM fixed and L.R. White embedded *Nicotiana plumbaginifolia* seeds showing the timing of protein deposition in the endosperm.



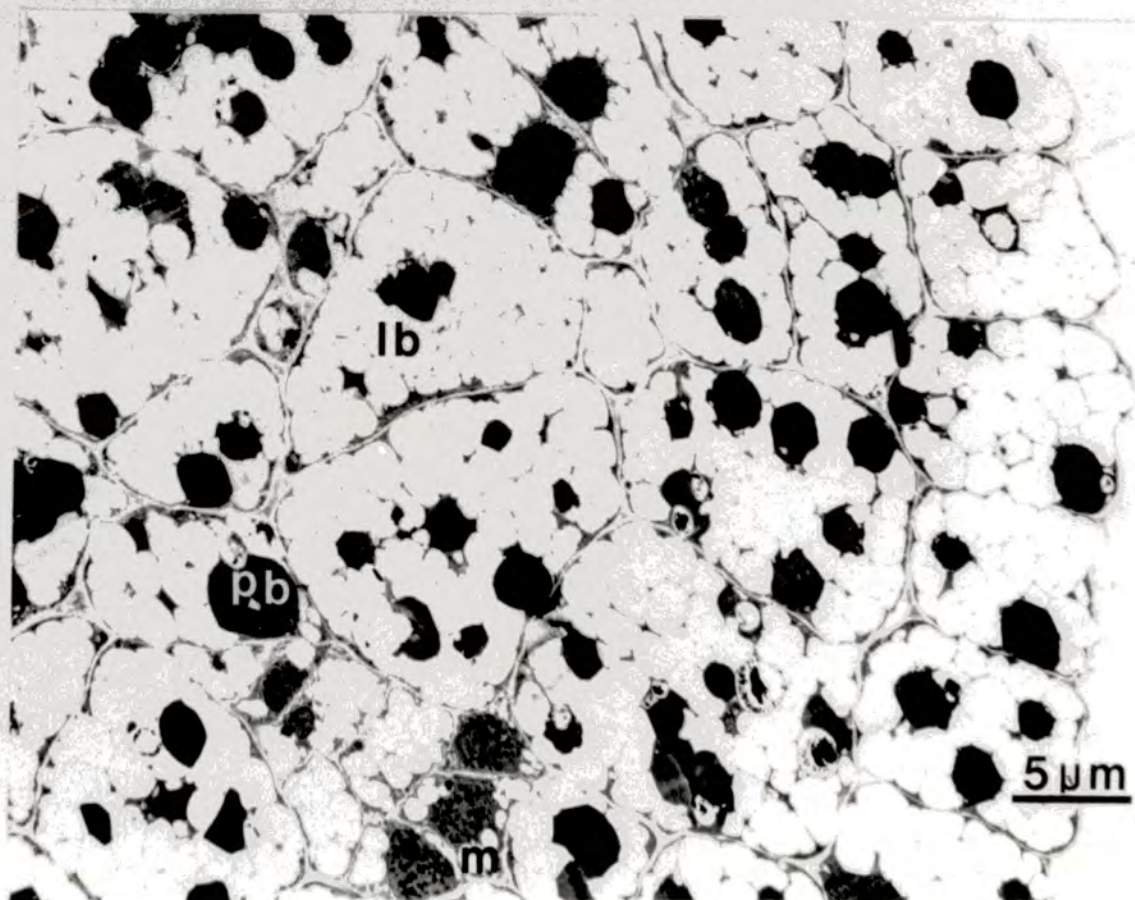
A. M-3. Darkly staining aggregates of protein (p) are contained within vacuoles (v).



B. M-2. The protein aggregates have grown to fill the vacuoles. Protein body (pb), lipid body (lb).

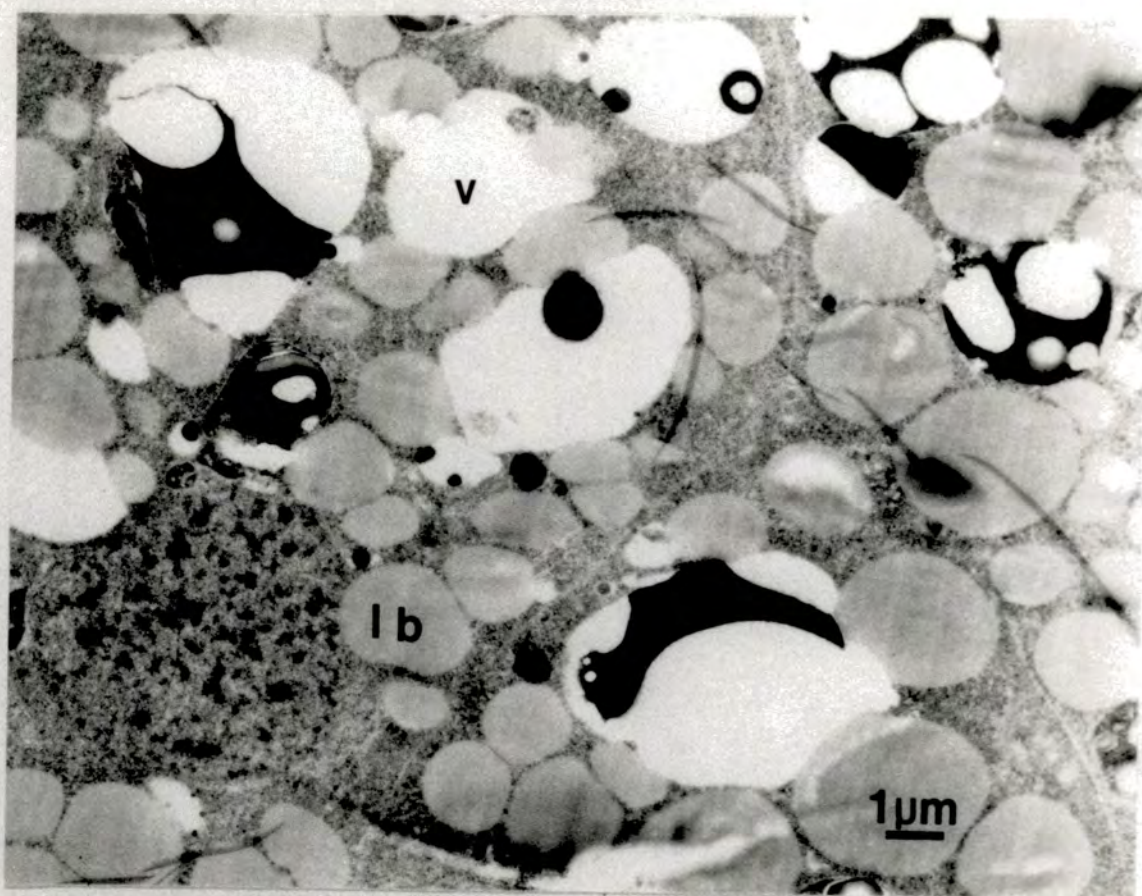


A. M-2. Darkly staining aggregates of protein (p) are contained within vacuoles (v).

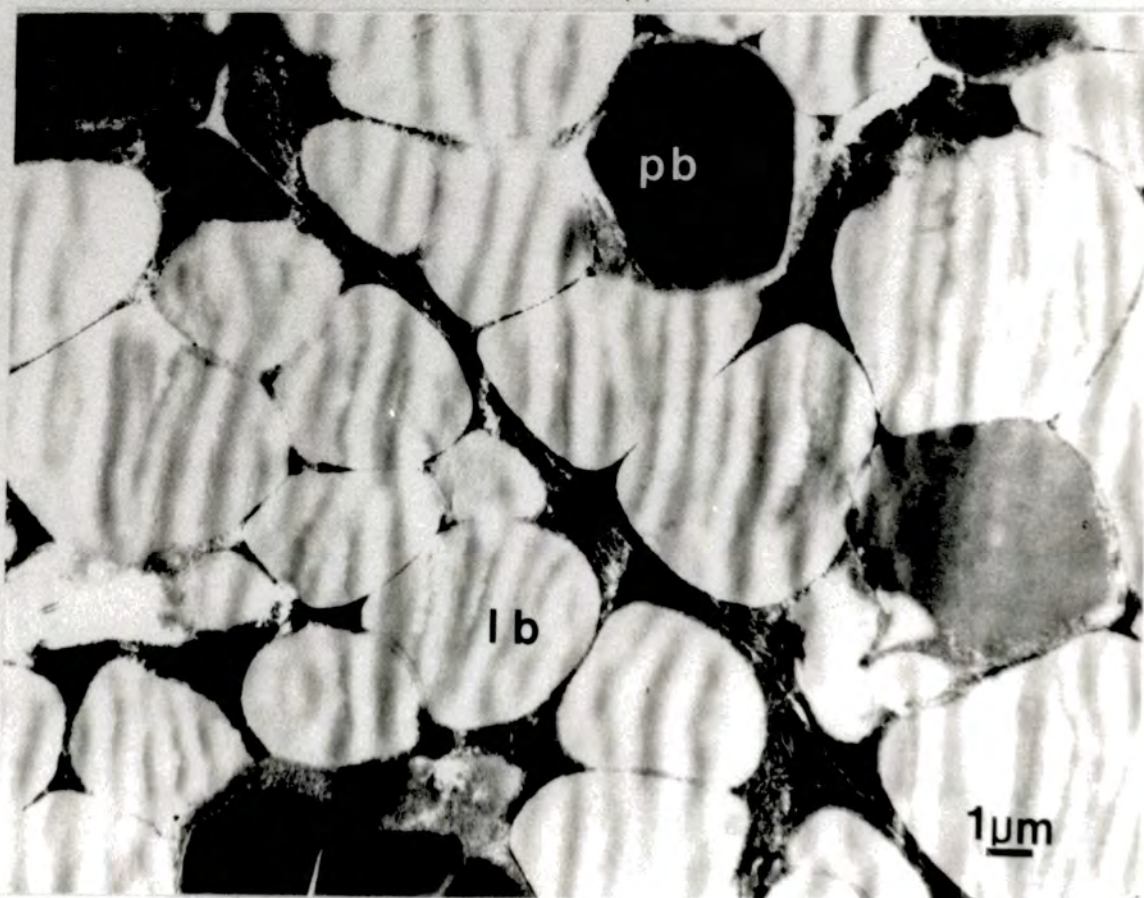


B. M-1. The protein aggregates have grown to fill the vacuoles. Protein body (pb), lipid body (lb), meristem (m).

Plate 7. Electron micrographs of the endosperm of ST fixed and Spurr embedded *Nicotiana plumbaginifolia* seeds.



A. M-3. Lipid bodies (lb) stain darker than vacuoles (v).



B. M-2. Protein bodies (pb), lipid bodies (lb).

it hard to distinguish their boundary membrane. Some vacuoles, however, remain unfilled even at maturity especially in the radicle. Hence it seems likely that the protein reserves are always in the form of membrane bounded protein bodies in both the embryo and the endosperm, and a range of their morphologies at maturity is shown in Plate 8.

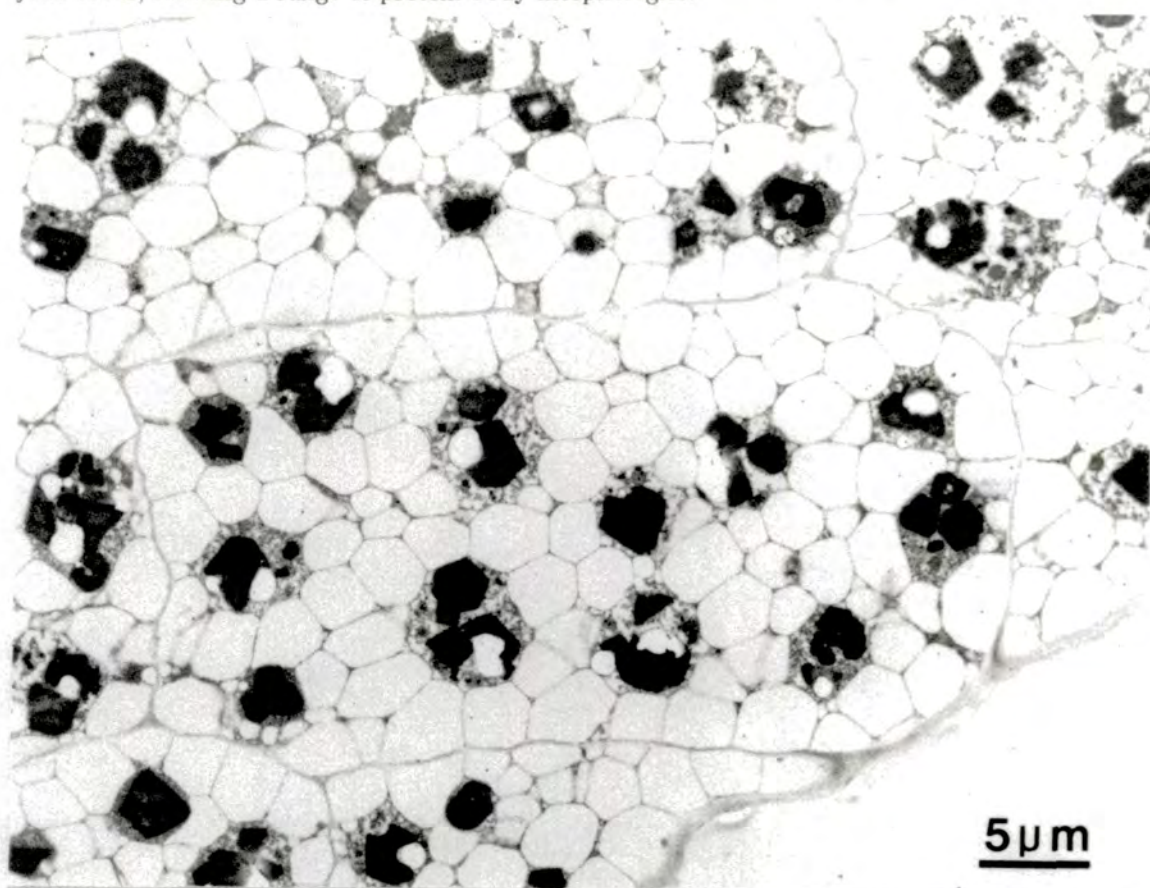
Assessment of a range of methods for immunocytochemical localization

(i) **Post-embedding localization at LM level-** Sections (approx. 1 μm thick) of IMM fixed, L.R. White embedded seeds were cut from blocks containing up to 9 seeds, which allowed for comparison of labelling intensities of different seeds within the same section. The use of polyclonal anti-legumin antisera, followed by a peroxidase-linked secondary antibody and DAB as a substrate which gave a brown stain, resulted in labelling of the protein bodies in both the embryo and endosperm of mature seeds (Plate 9a). However, the intensity of the brown staining was variable, sometimes being ambiguously low, and no consistent differences could be detected in the labelling in non-transformed and transformed seeds. When DAB was used with the addition of cobalt chloride, a black stain was produced which was more clearly visible, but the background of non-specific stain production was higher, and this was also the case when 4-chloro-1-naphthol was used as the substrate.

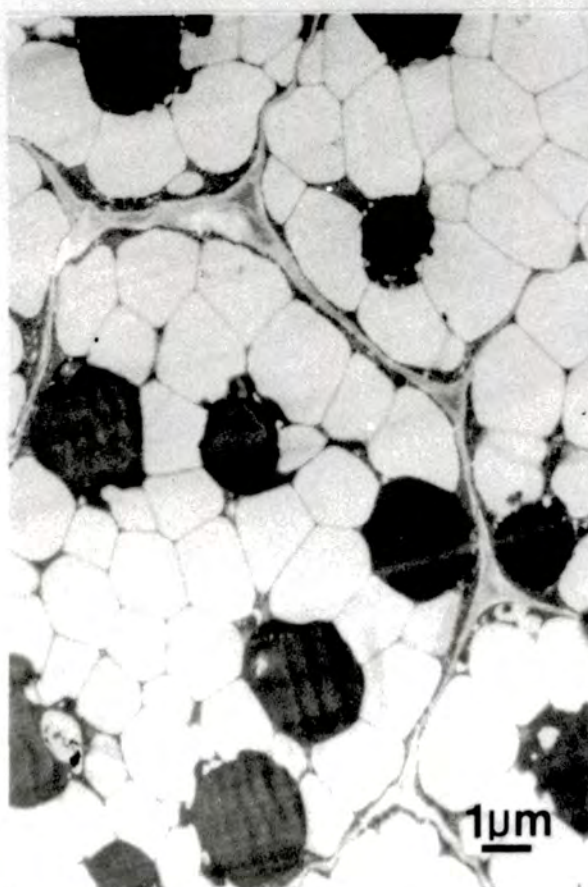
The fluorescence-labelled secondary antibody did not produce any detectable labelling, which could be distinguished under the examination conditions. The use of the same reagent in other situations also failed to produce any detectable labelling, thus it was presumed that this batch of the reagent was defective.

Polyclonal anti-legumin antisera, visualized by the silver enhancement of gold-labelled secondary antibodies gave rise to labelling of protein bodies in both the embryo and endosperm of mature seeds. The labelling, shown in Plate 9b, was of

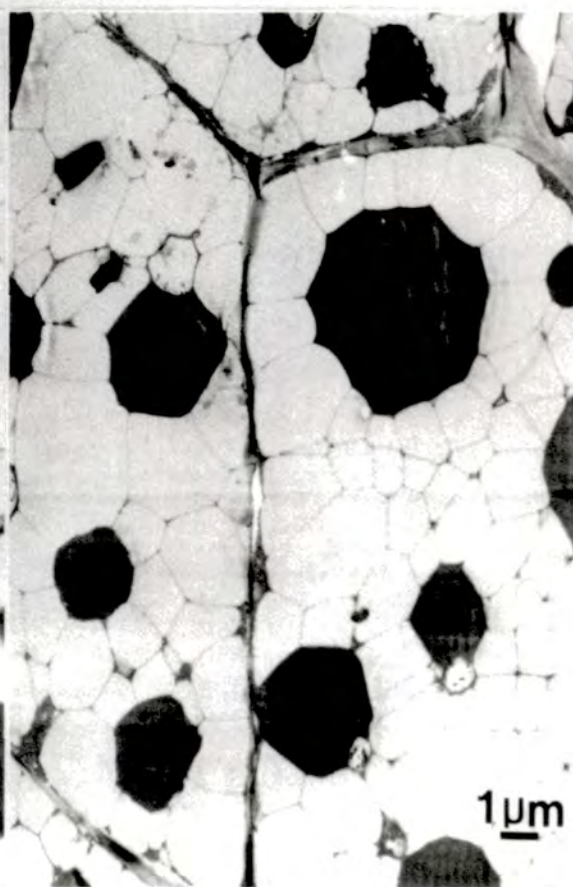
Plate 8. Electron micrographs of IMM fixed and L.R. White embedded mature *Nicotiana plumbaginifolia* seeds, showing a range of protein body morphologies.




A. Embryo, radicle.



B. Embryo, cotyledon.

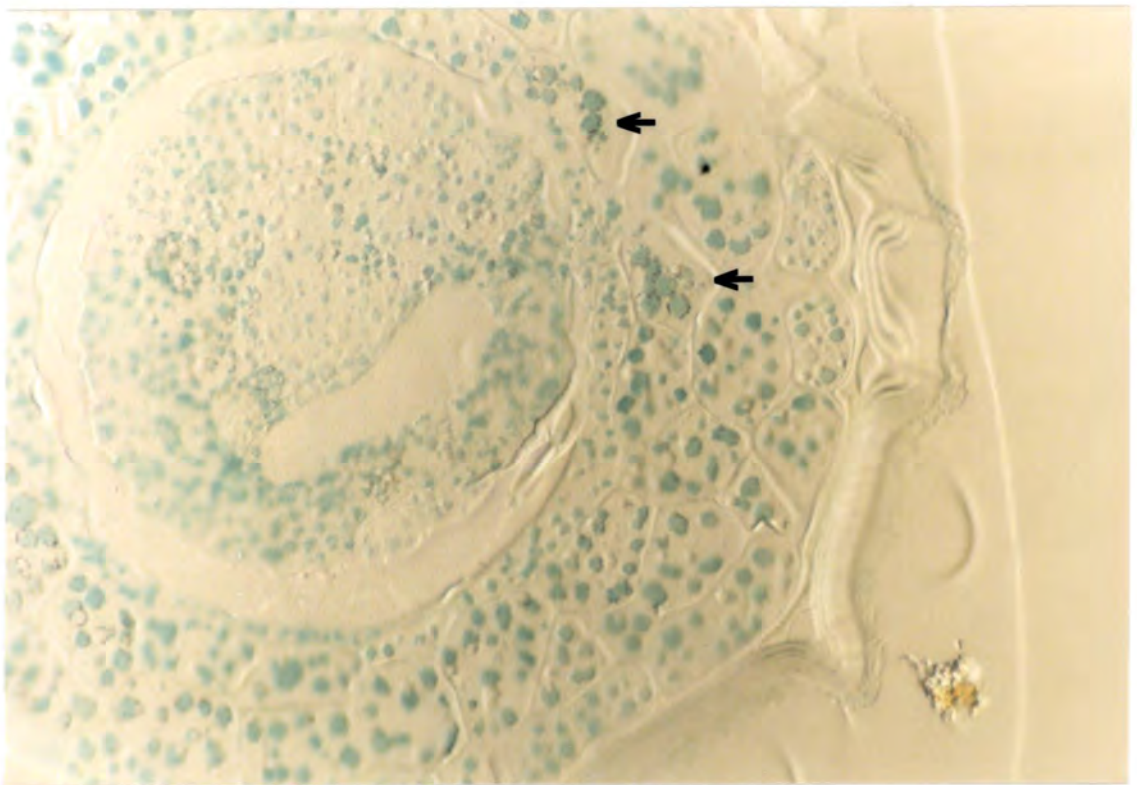


C. Endosperm.

Plate 9. Post-embedding immunocytochemical localization of legumin at LM level; sections of IMM fixed and L.R. White embedded seed, with Nomarski optics. Scale bar = 50 μm . 



A. Peroxidase-linked secondary antibody with DAB as a substrate, causing brown stain deposition over the protein bodies.



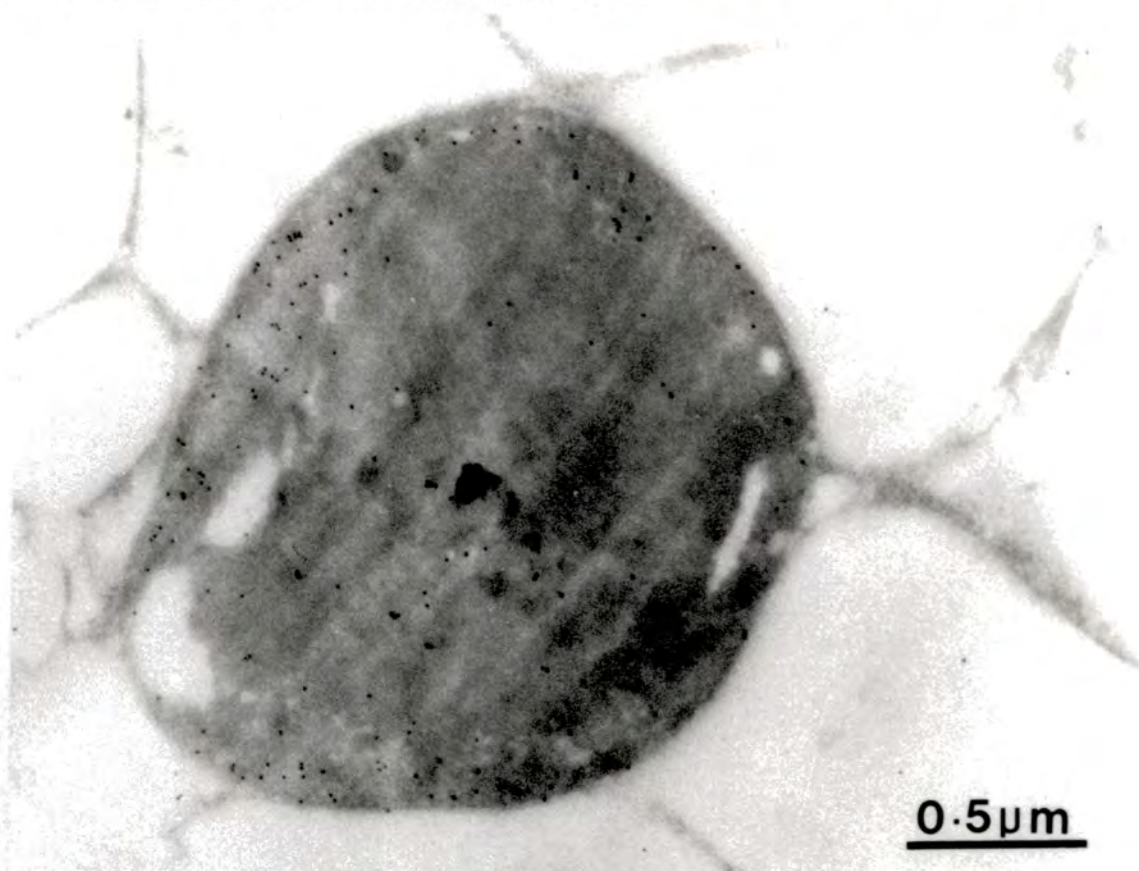
B. Gold-labelled secondary antibody with silver enhancement, tissue counter-stained with light green. Arrows indicate areas where labelling of the protein bodies can be most easily observed

a low intensity and could only be seen clearly at higher magnifications, but there was very little non-specific staining and the labelling could be identified with confidence. Again, however, there was no consistent difference in the labelling of transformed and non-transformed seeds.

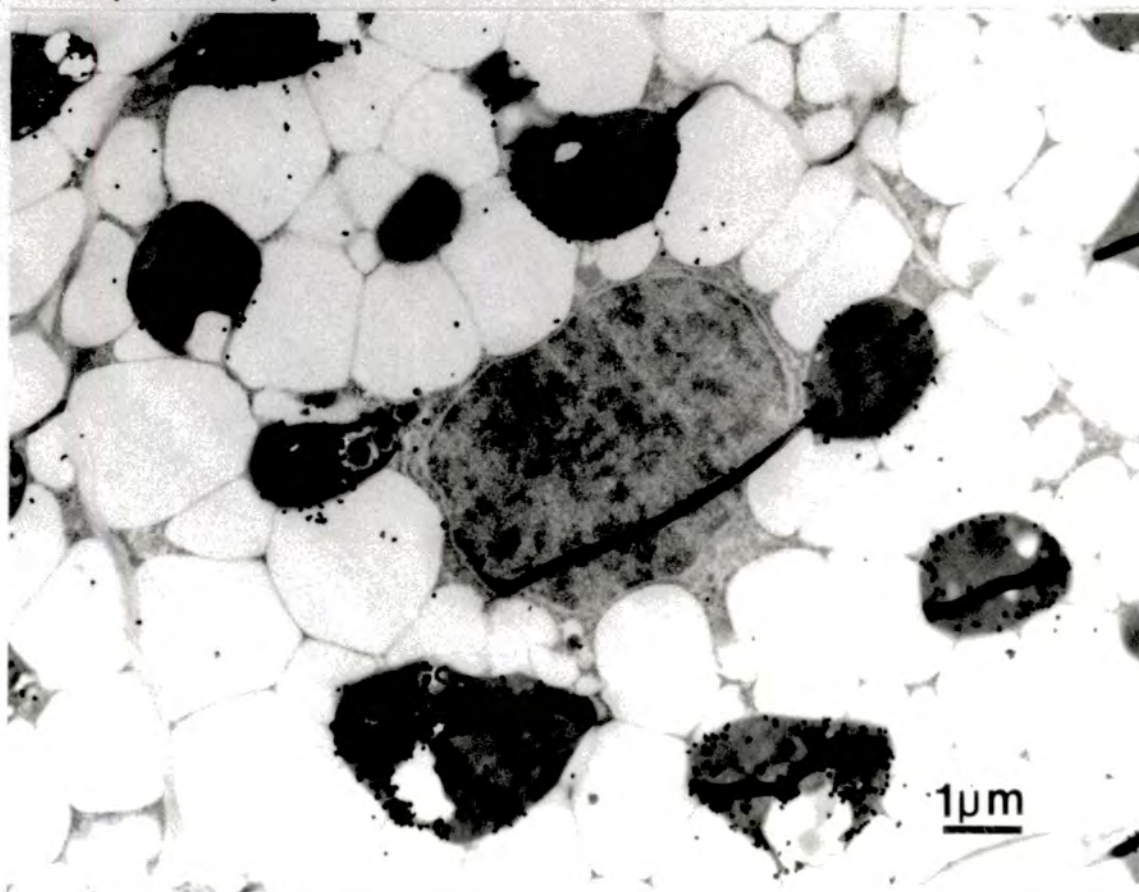
(ii) **Post-embedding localization at EM level-** Application of the post-embedding immunocytochemical technique for ultrastructural localization in sections of resin embedded tissue showed that the protein bodies were labelled in a similar way to that observed at the LM level. Plate 10a shows a protein body labelled with secondary antibodies adsorbed onto colloidal gold particles with a mean diameter of 10 nm. The labelling was specific to the protein bodies with only a low background of non-specific labelling on other cell components, except in the seed coat where there was a high level of non-specific labelling. Secondary antibody adsorbed onto smaller sized gold colloid particles (5 nm) produced a higher level of labelling than the larger particles but require higher magnification to be visible. The smaller particles were also more difficult to distinguish from the grainy texture of protein stained with both uranyl acetate and lead citrate. In sections examined without contrast staining the labelling with 10 nm particles was more distinct, but the ultrastructural detail of the tissue was hard to distinguish. The use of 10 nm particles and staining with uranyl acetate only, produced a good compromise of labelling intensity, visibility and ultrastructural details.

Plate 10b shows the labelling produced by the silver enhancement of the colloidal gold particles of the secondary antibody complex. This approach allowed the use of 5 nm particles to maximise the labelling intensity. The numerous small particles were then enhanced to a large enough size to be visible at low magnification in the EM. Sections were stained with both uranyl acetate and lead citrate, revealing clear ultrastructural details.

Plate 10. Post-embedding immunocytochemical localization of legumin at EM level; in sections of IMM fixed and L.R. White embedded transformed seed.



A. Gold-labelled secondary antibody, with a 10 nm mean particle diameter, tissue counter-stained with uranyl acetate only.



B. Gold-labelled secondary antibody (5 nm) with silver enhancement, tissue counter-stained with uranyl acetate and lead citrate.

The labelling at EM level was interpreted more reliably, than that at the LM level, as any labelling associated with non-specific staining or physical damage to the section could easily be identified and discounted. Thin sections suitable for EM examination could only be cut from a block face of much smaller area than was possible for LM sections, and so sections containing only 1 or 2 seeds could be cut.

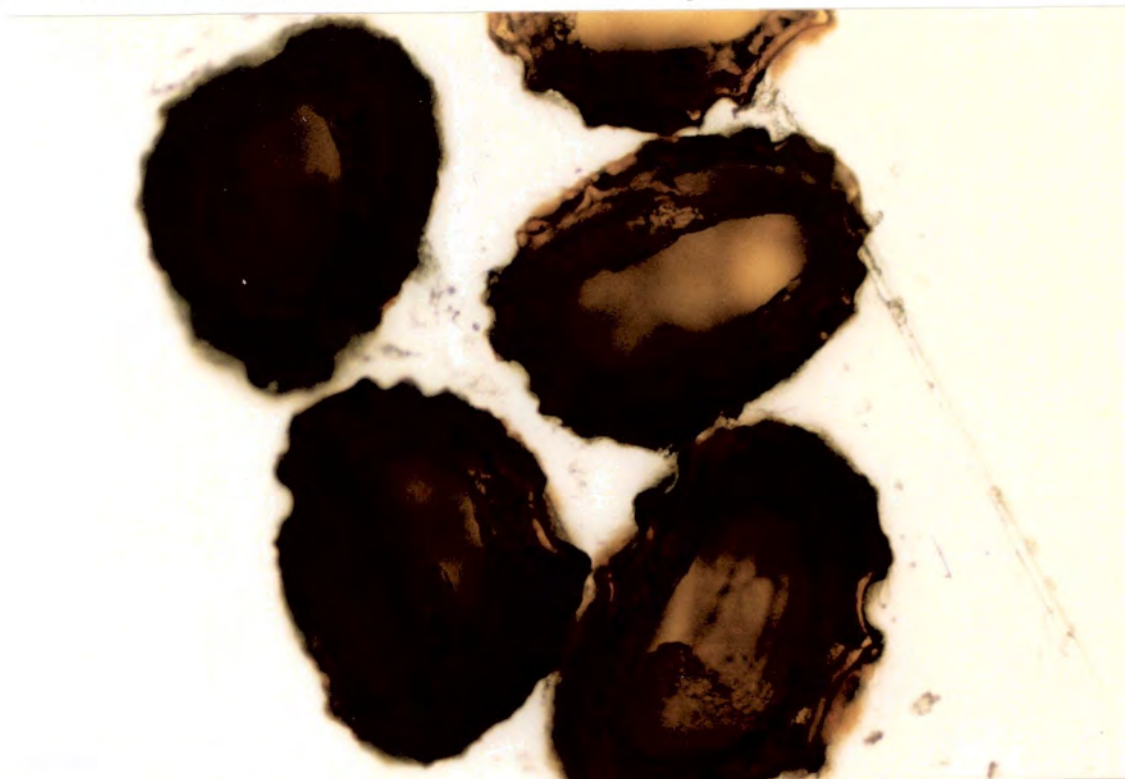
(iii) **Pre-embedding localization-** Non-embedded seeds were cut and fixed briefly prior to immuno-localization using silver intensification (Plate 11a) or DAB labelling (Plate 11b,c). The protein bodies were labelled in a similar pattern to that produced in the post-embedded material. Examination of the unsectioned material at the LM level was, however, hampered by the difficulty of achieving good illumination, because of the thickness of the material, and hence the quality of the images observed was poor. Although some labelling was achieved using silver enhancement, it was not as readily distinguishable as the DAB labelling under the poor illumination conditions. Immuno-localization using unfixed tissue, either with DAB or with silver enhancement, generally gave a low intensity of labelling, but the results could not be reproduced reliably with either staining protocol.

When the same fixed and unfixed materials were used in a pre-embedding procedure, using a joint labelling with both peroxidase/DAB and protein A-gold, the LM and EM level labelling was found only at a low intensity at the cut surface of the seeds. It was difficult to find an appropriate orientation of the block in order to cut sections through the narrow band of labelled material.

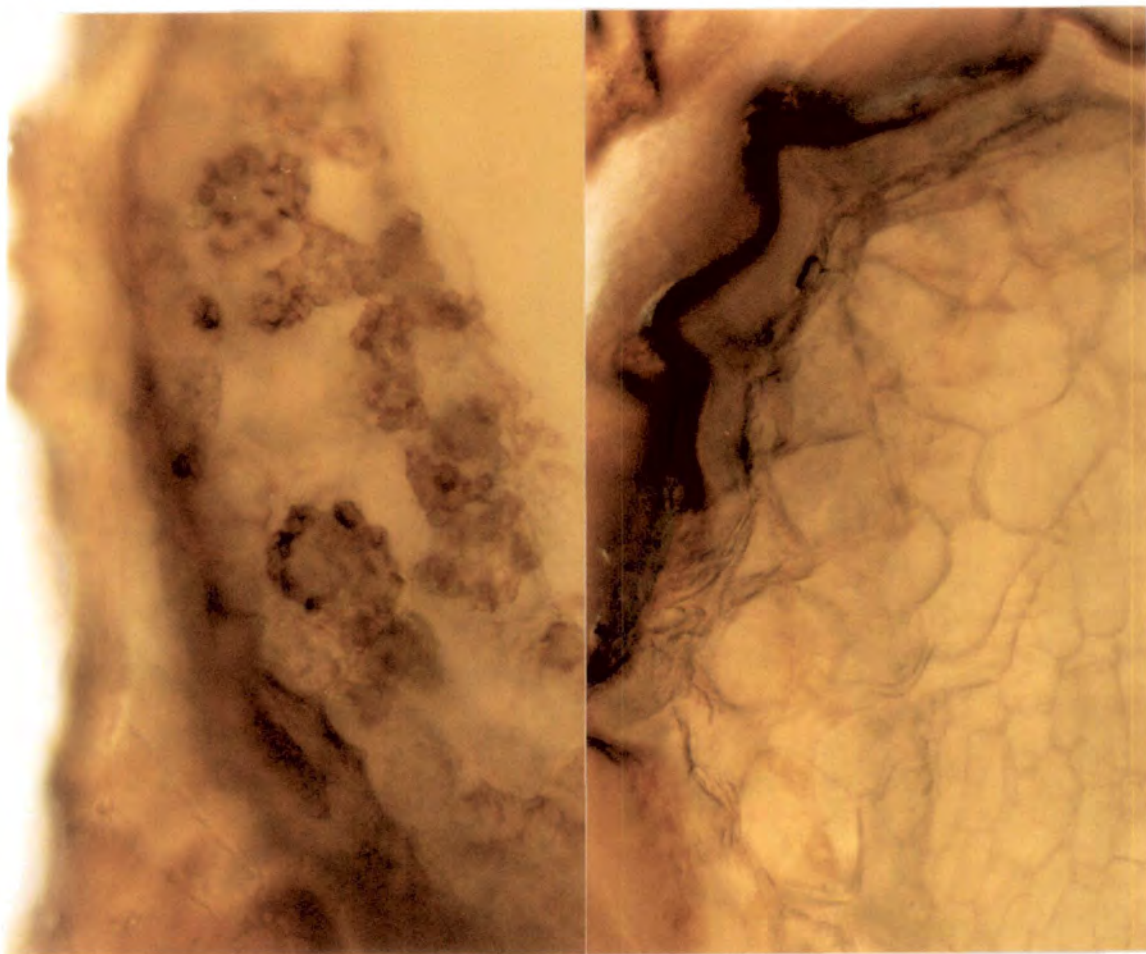
Development of reliable immunocytochemical localization techniques

From the results achieved with the range of immunocytochemical techniques de-

Plate 11. Pre-embedding immunocytochemical localization of legumin at LM level; in the cut surface of IMM fixed seeds, immobilized in wax, no counter-staining.



A. Gold-labelled secondary antibody with silver enhancement, at low power, showing a group of seeds, with variable labelling. Scale bar = 0.2 mm. —



B,C. Peroxidase-linked secondary antibody, with DAB. Scale bar = 25 μ m. —

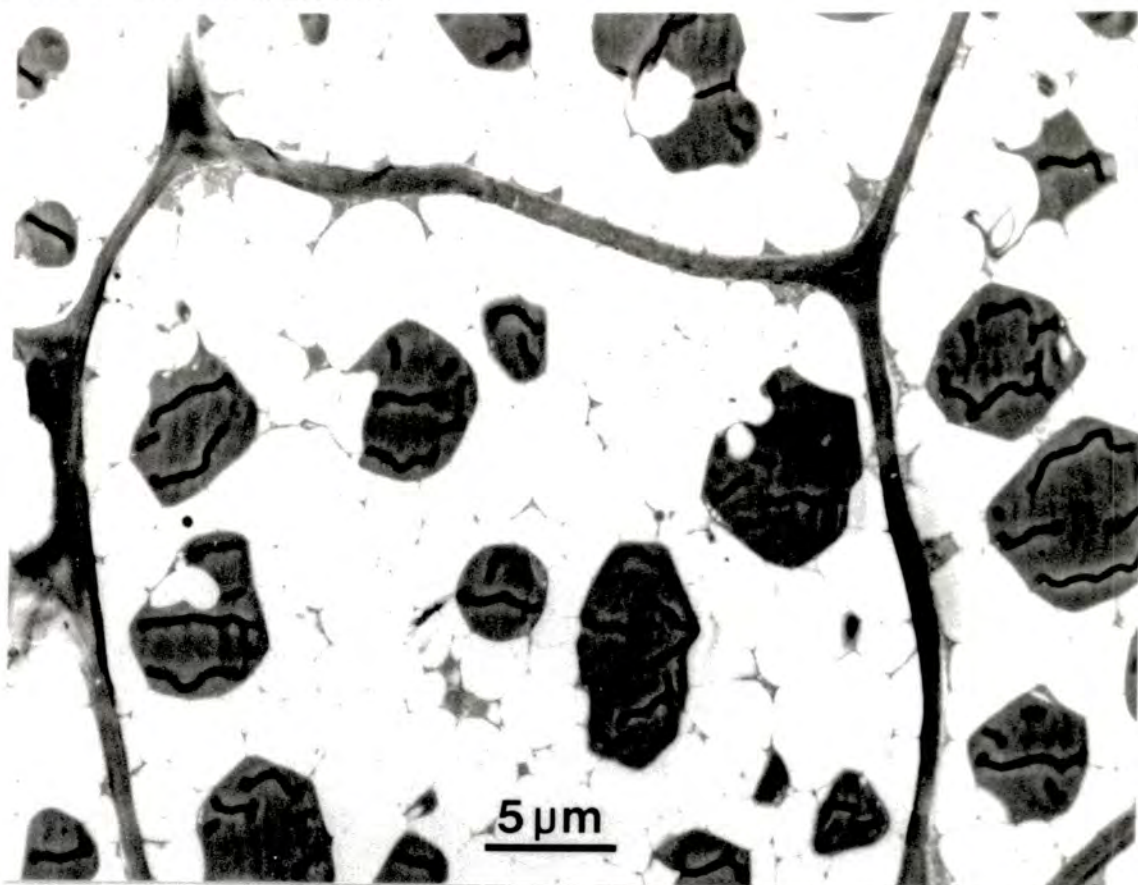
B. High power showing labelling of protein bodies. **C.** High power showing non-labelled protein bodies.

scribed above, it became clear that although labelling could be achieved, consistent differences between the transformed and the non-transformed seeds were not being achieved. This lack of specificity could have been caused in a number of ways, but reference to the results of control experiments eliminated some possibilities. The lack of any labelling when buffer was used instead of antibody for the primary incubation, as can be seen in Plate 12a, demonstrates that there is no significant non-specific labelling caused by any of the other reagents in the absence of the primary antibody. The low level of labelling resulting from the use of a pre-immune serum instead of a primary antiserum (raised in the same species), as seen in Plate 12b, indicates that the lack of specificity results from the antibodies produced in response to the injection of legumin rather than any other components of the serum. Thus the most likely cause of this lack of specificity is the presence of an endogenous *Nicotiana plumbaginifolia* protein which is also recognised to some extent by antibodies raised against *Pisum* legumin.

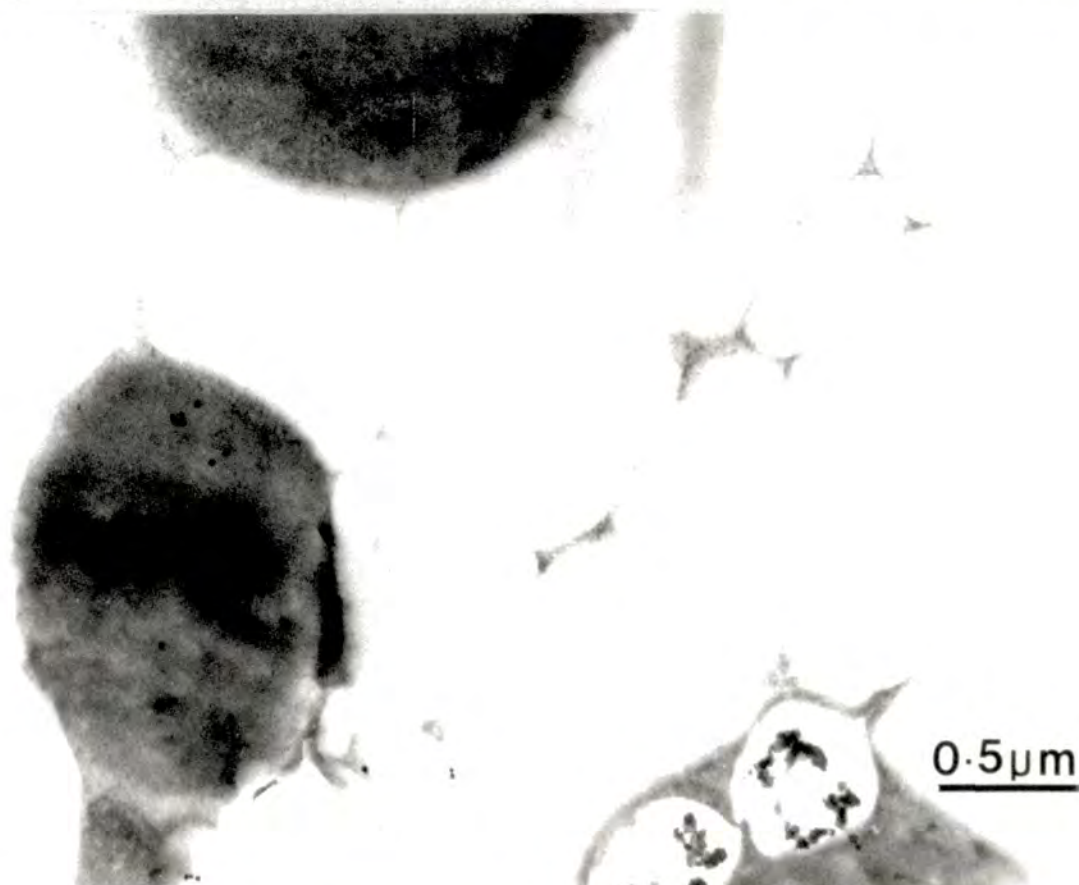
Results from ELISA of single seeds (Kang *et al* 1988) had clearly demonstrated that first generation transformed plants produced a mixture of transformed and non-transformed seeds. As it was not possible to determine the phenotype of a seed before attempting immunocytochemical localization, it was necessary to use a large sample size of seeds to increase the probability of working with a transformed seed. This could most readily be achieved at the LM level where a larger surface area of block face could be sectioned, allowing up to 9 seeds to be investigated simultaneously.

(i) Physical and chemical factors- A range of physical and chemical factors were investigated to determine their contribution to the differential in labelling between transformed and non-transformed seeds. There was no apparent enhancement of the differential labelling amongst the 9 seeds, when the various localization steps were performed at either 4 °C or 37 °C rather than at room

Plate 12. Immunocytochemical localization control experiments in IMM fixed and L.R. White embedded seed sections at EM level.



A. Primary incubation with buffer containing no antibody, followed by gold-labelled secondary antibody with silver enhancement; tissue counter-stained with uranyl acetate and lead citrate. There is no significant labelling.



B. Primary incubation with pre-immune serum, followed by gold-labelled secondary antibody; tissue counter-stained with uranyl acetate only. There is a low level of non-specific labelling.

temperature. Similarly, there was no enhancement of the differential in labelling caused by any of a range of different incubation times and dilution factors of the reagents, although the overall labelling intensity increased with higher temperatures, longer incubations and lower dilution factors of the reagents. Various different washing protocols were used between the localization steps, but none was found which enhanced the differential staining within the seed sample.

A variety of buffers based on Tris/HCl and PBS at a range of pHs, salt concentrations, with or without a range of dilutions of added detergent (Tween-20) all gave a similar lack of differential in labelling of the seeds. The incubation with BSA intended to block non-specific binding sites was useful to reduce the labelling of the seed coats, but did not enhance the differential between seeds. Results with human pre-immune serum as a blocking agent were similar. Poly-clonal antisera from more than one rabbit was used with or without affinity purification and antiserum from a mouse, but none gave any better differential labelling than the others.

These various factors were investigated in isolation and in various combinations, with reference to appropriate control experiments. Although these factors were clearly important in influencing the overall labelling intensity and its specificity to protein bodies, there was no apparent influence on the relative labelling intensity in different seeds.

During the course of this work it became increasingly clear that working with seeds of unknown phenotype was adding significantly to the difficulty of developing suitable localization techniques. Labelling intensities observed at the LM level could not be assessed with as great a precision as those at the EM level, but development of a method at the EM level would require the simultaneous use of a large number of sections in order to achieve as good a sample size as that of the 9 seeds at the LM level.

(ii) **The use of seeds of known phenotype-** Individual mature seeds from a transformed plant were phenotypically characterised by an ELISA of one half of each seed (Kang, personal communication); the corresponding other halves were IMM fixed and embedded. Two seeds were identified by ELISA as high legumin expressors, but when sections of the other halves were examined the embryos were missing in both cases. Immunocytochemical localization of legumin at both LM and EM levels produced similar results in the endosperm to those observed at LM level with the 9 seeds, with no clear enhancement of labelling when compared with non-transformed endosperm tissue.

(iii) **Identification of a homozygous legumin positive line-** A second generation transformed plant was selected on the basis of the results of ELISA performed on pooled seed extracts which indicated a high level of *Pisum* legumin expression (Kang, personal communication). Single seed ELISA was performed using 32 of its seeds and these were compared with 8 seeds from a non-transformed plant and 8 assays without any seeds. The values gained for the 8 non-transformed seeds were higher than the 8 assays with no seeds, indicating that some non-specific binding also occurs in the ELISA. Of the 32 seeds from the transformed plant, 29 produced a result that was significantly higher (ie more than the mean plus 2.0 standard deviations) than the non-transformed seeds. This result was consistent with the possibility that all the seeds were of legumin expressing phenotype, produced by selfing a parent plant with a homozygous legumin positive genotype.

Seeds from this plant were fixed (IMM) and embedded in L.R. White resin, and localization was attempted as before at both EM and LM levels. The results, when compared with labelling of non-transformed tissue, confirmed that there were no physical and chemical factors in the immunolocalization protocols, which, in isolation or in combination, could enhance the labelling differential between

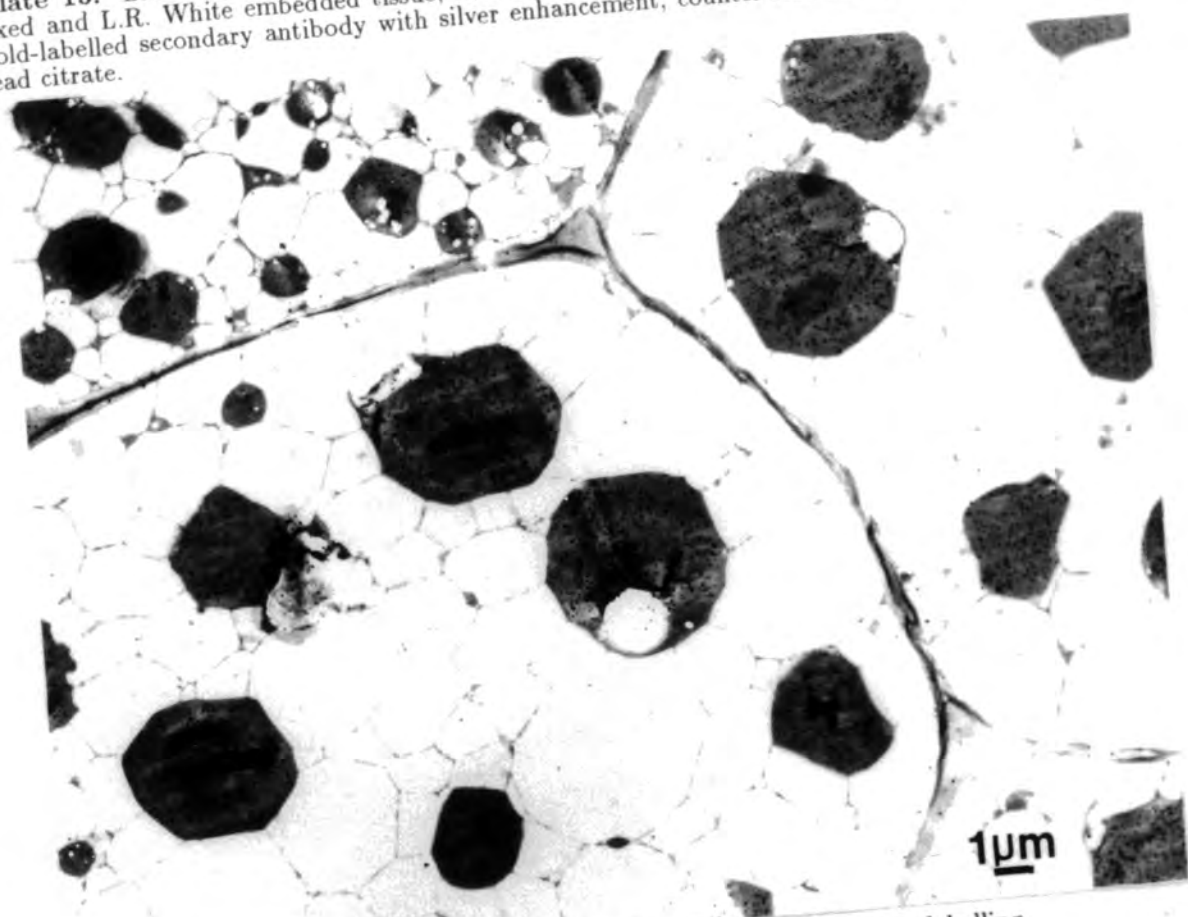
transformed and non-transformed seeds. Thus the remaining option was to try different ways of modifying the primary antibody in order to achieve a suitable differential in the labelling of transformed and non-transformed seeds.

(iv) **Mono-clonal primary antibody-** When a range of mono-clonal mouse antibodies were used as the primary antibody none was found which was able to give a high enough labelling intensity in transformed seeds to be significant in comparison with the low level of non-specific labelling in non-transformed seeds.

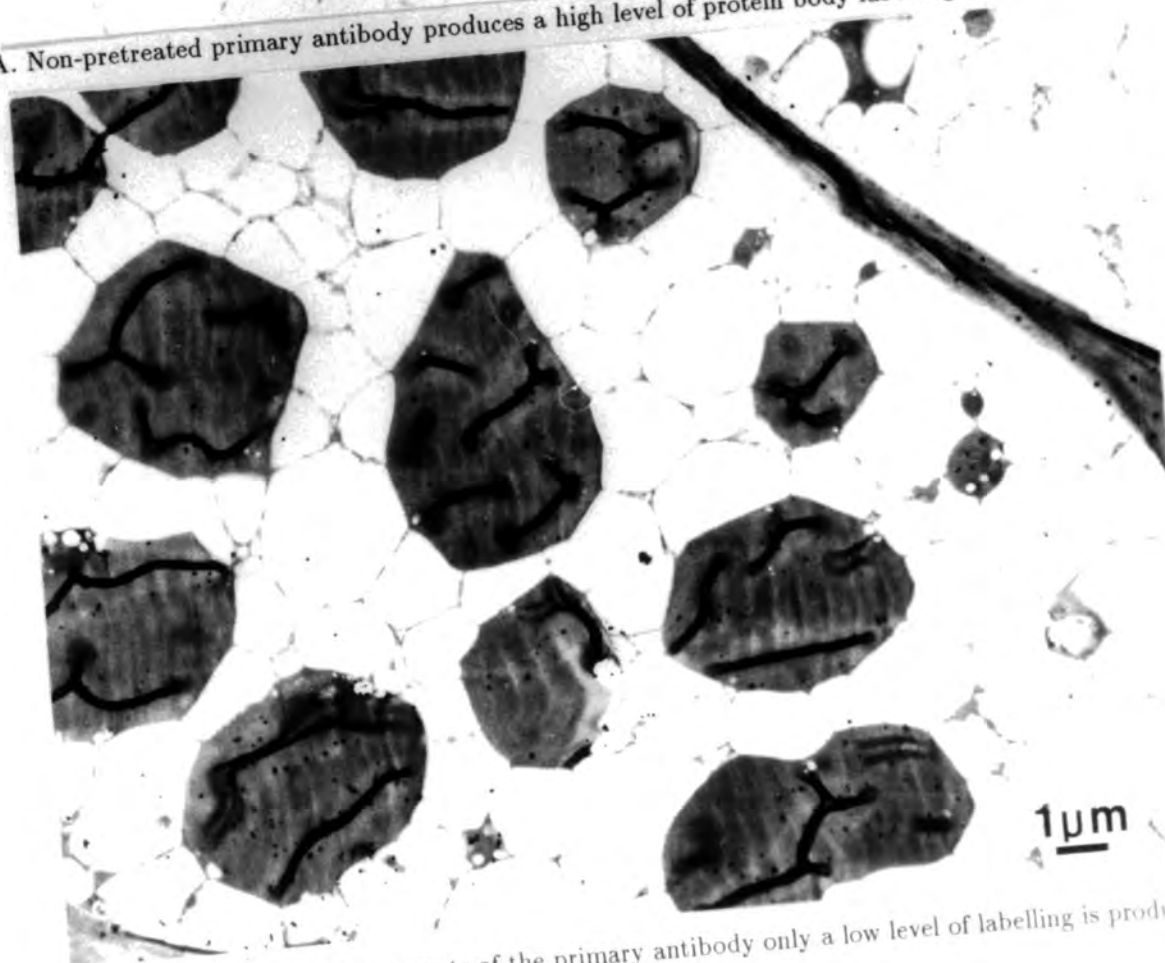
(v) **Pretreatment of the primary antibody-** Pretreatment of the polyclonal anti-legumin antibodies, with non-transformed seed extract, caused a great reduction in the seed coat labelling, and sometimes gave slightly different intensities of protein body labelling amongst the 9 seeds in one section. However results were not reproducible and in control experiments, using non-transformed seeds, the protein bodies were still labelled, often with equal intensity to those of the transformed seeds.

Repeated pretreatments of the primary antibody produced gradual improvement in the differential between transformed and non-transformed seed labelling. After 9 successive pretreatments it was possible to produce consistent and unambiguous labelling of transformed seeds that was significantly more intense than that in the non-transformed seeds. Plate 13a shows the labelling in the endosperm of non-transformed seeds, produced with a non-pretreated primary antibody and Plate 13b shows the reduction in labelling intensity in the same tissue caused by 9 pretreatments. The labelling in the embryo of non-transformed seeds is similarly shown in Plate 14a, without pretreatment and in Plate 14b, with 9 pretreatments. The non-pretreated antibody gives significant labelling which must indicate the occurrence of an endogenous protein in the non-transformed phenotype, whilst

Plate 13. Labelling in the endosperm of non-transformed seed at EM level; in sections of IMM fixed and L.R. White embedded tissue, incubated with anti-legumin primary antibody followed by gold-labelled secondary antibody with silver enhancement, counter-stained with uranyl acetate and lead citrate.

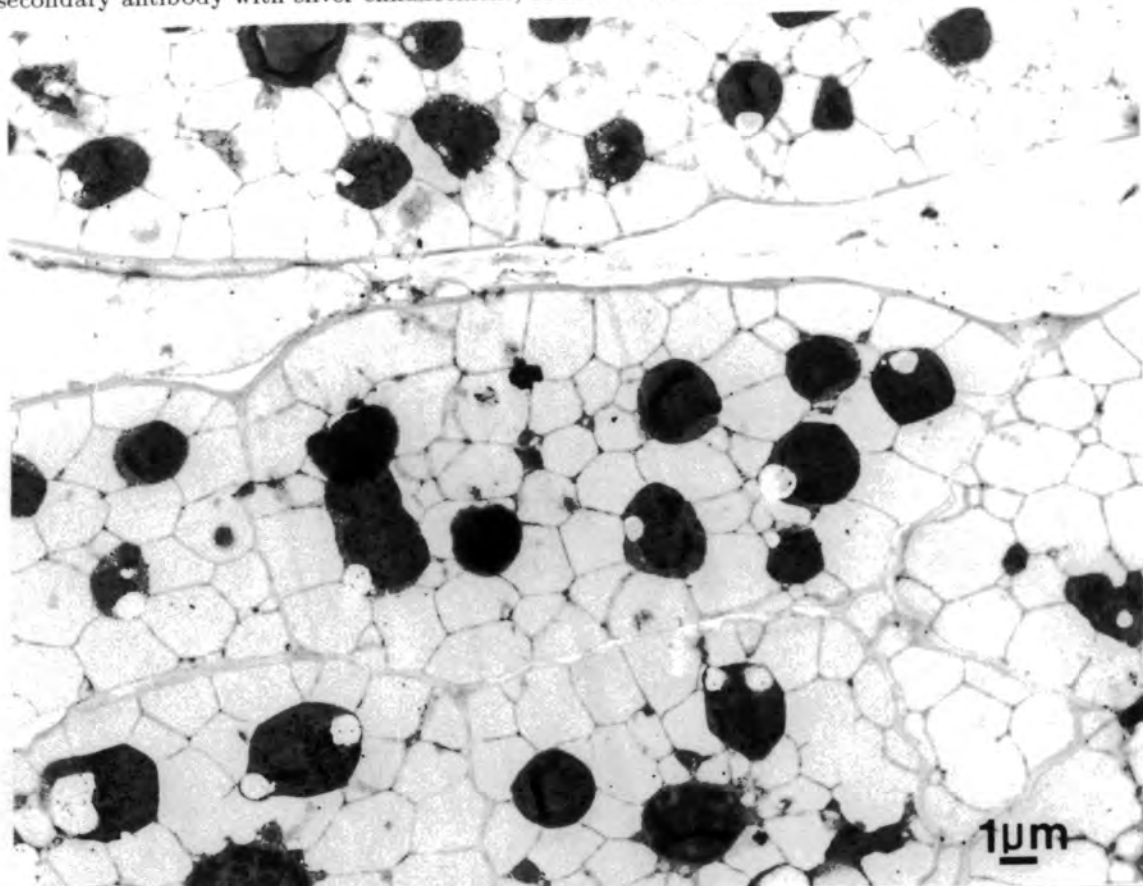


A. Non-pretreated primary antibody produces a high level of protein body labelling.

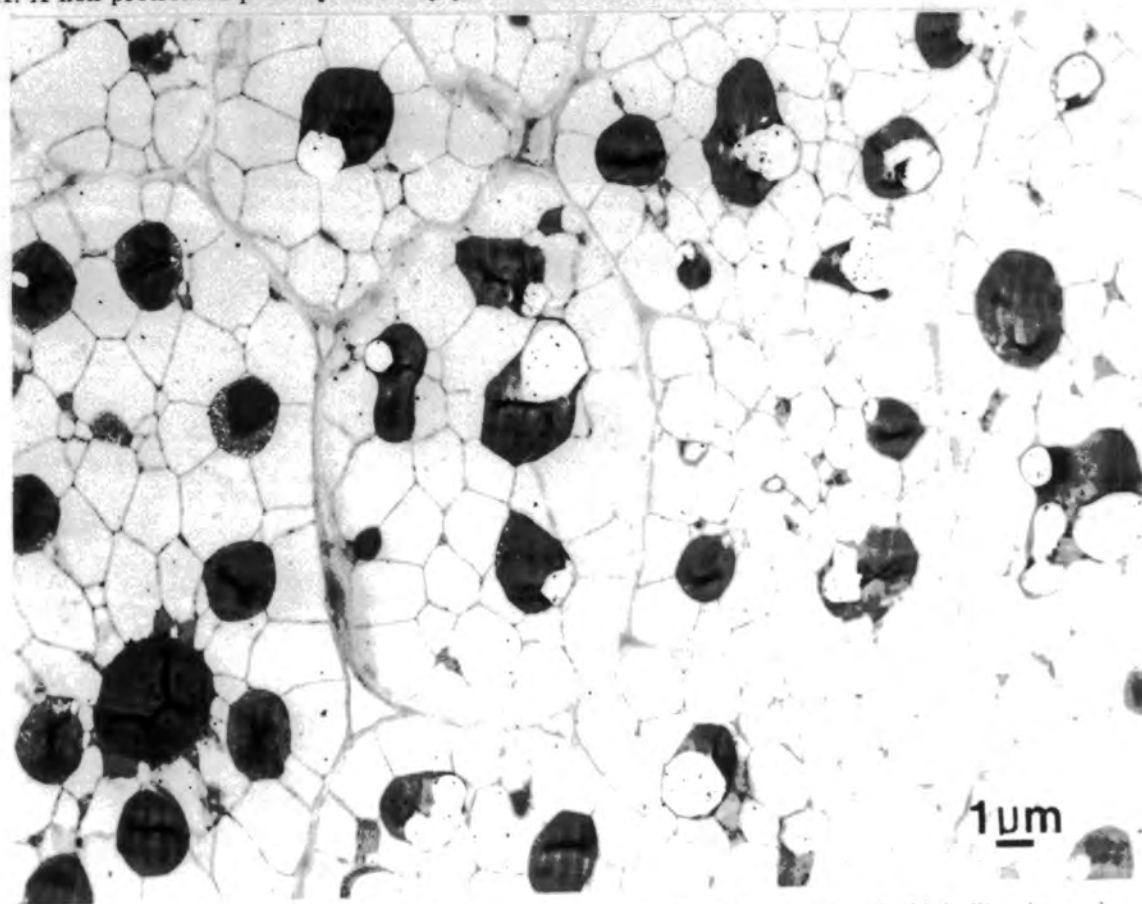


B. After 9 successive pretreatments of the primary antibody only a low level of labelling is produced.

Plate 14. Labelling in the embryo of non-transformed seed at EM level; in sections of IMM fixed and L.R. White embedded tissue, incubated with anti-legumin primary antibody, followed by gold-labelled secondary antibody with silver enhancement, counter-stained with uranyl acetate and lead citrate.



A. A non-pretreated primary antibody produces a high level of protein body labelling.



B. After 9 successive pretreatments of the primary antibody only a low level of labelling is produced. There is also significant non-specific labelling of the formvar exposed by holes in the section.

the pretreated antibody does not give a significant level of labelling. The Western blot of seed extracts of pea and non-transformed *Nicotiana plumbaginifolia* and purified legumin clearly demonstrated the effect of pretreatment of the primary antibody, Plate 15; protein bands from *Nicotiana* which are hardly visible after probing with the pretreated antibody are more intense after further probing with non-pretreated antibody. In transformed seeds, the pretreated antibody still produced significant labelling, as can be seen in Plate 16.

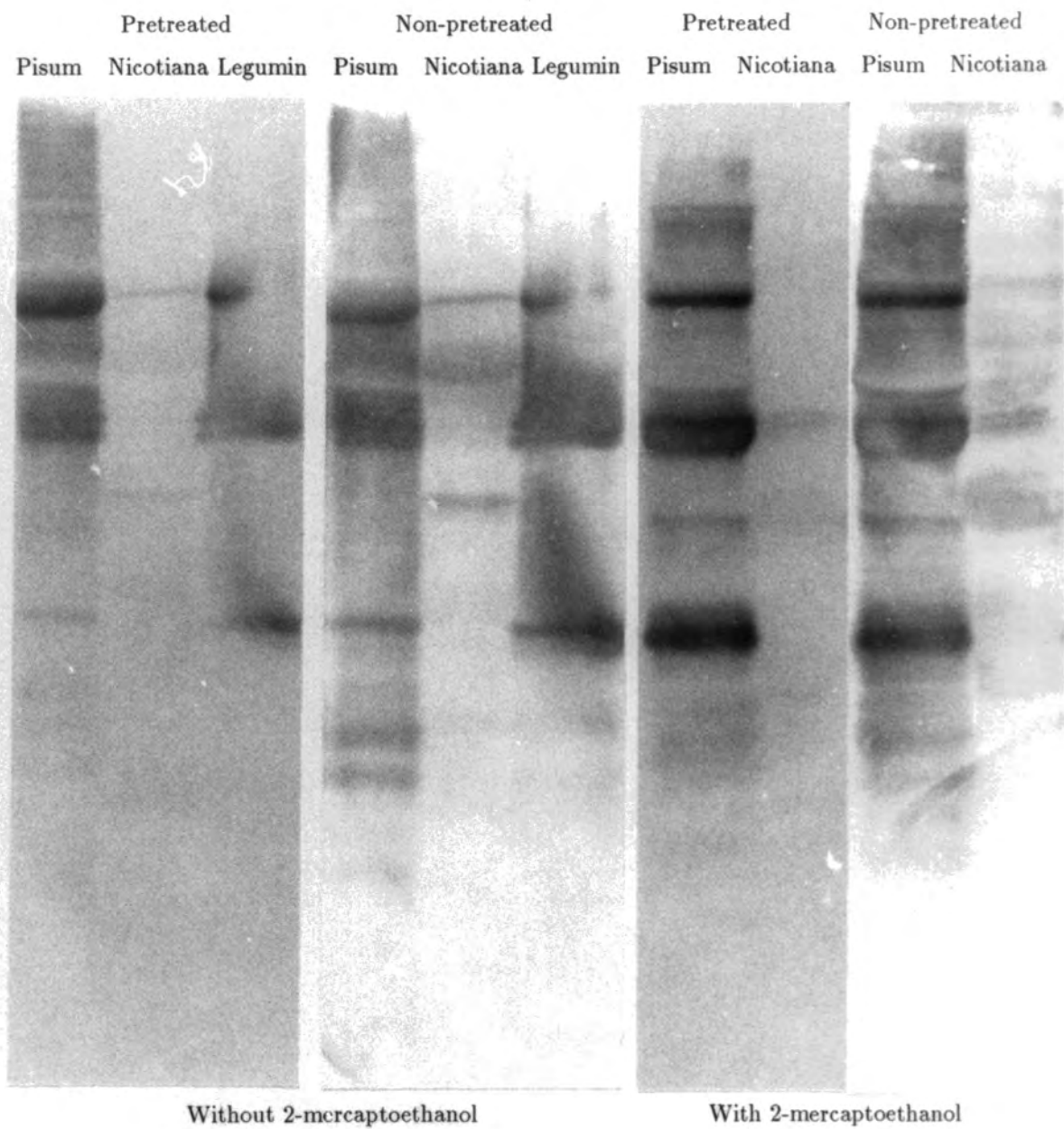
The distribution of *Pisum* legumin in mature transgenic seeds

The protein body labelling observed in the cotyledons of transformed seeds with the 9 times pretreated antibody consisted of a very low level of labelling all over the protein and a distinct band of intense labelling around the outside of the protein aggregates. No equivalent band of intense labelling was ever observed in any non-transformed seeds. These results were consistently reproducible. Thus this pattern of labelling was an un-ambiguous indication of the distribution of the transgenically expressed *Pisum* legumin.

Some protein bodies were observed in which the protein appeared to be less dense in the peripheral region, as shown in Plate 10a, and the labelling was associated with this region. Protein bodies with more than one dense, crystalline region were observed; in these the heavy labelling was associated with all of the less dense protein rather than just the periphery of the aggregate. Tangential sections through the periphery of protein aggregates were always found to be heavily labelled, whilst near serial sections through the main body of the same aggregate had a peripheral distribution of heavy labelling.

This specific labelling of *Pisum* legumin was observed in cells of the cotyledons and embryo axis adjacent to the cotyledons. Proto-vascular tissue, the radicle and epidermal cells did not have significantly heavier labelling than the background in

Plate 15. Western blot showing the effect of pretreatment of the primary antibody.



Protein bands in the Nicotiana extract appear more intense after screening with the non-pretreated primary antibody.

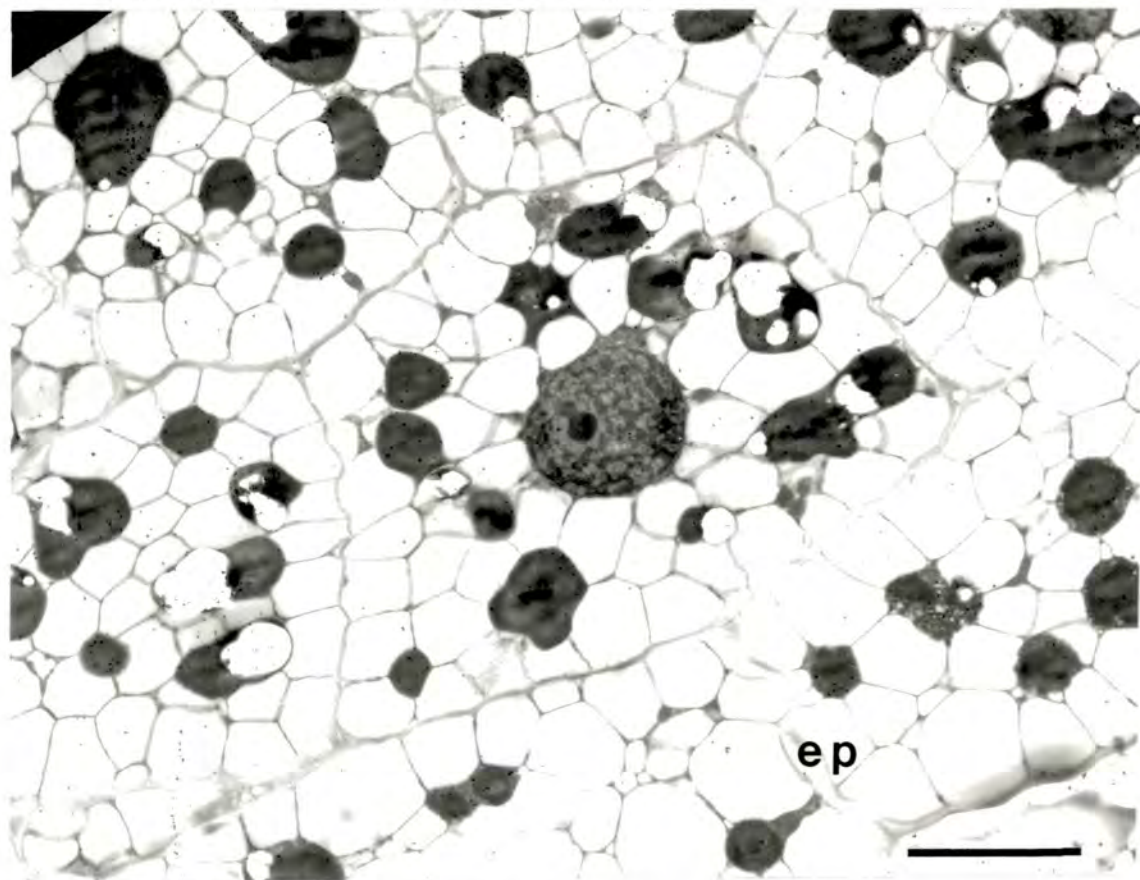
the non-transformed seeds. Plate 16a shows the labelling in a mature cotyledon which may be compared with that in the axis (Plate 16b). Immuno-labelling of the radicle of transformed seeds is illustrated in Plate 17a; it is not significantly more intense than that observed in non-transformed seeds.

Within the mature endosperm a few cells could be identified which showed the peripheral pattern of labelling, characteristic of specific *Pisum* legumin immunolocalization within embryo tissues. The intensity of labelling in the endosperm was not as great as that seen in the embryo, and in most cases the labelling was insufficient to be clearly identified. Also the background of non-specific labelling of protein bodies in non-transformed seeds was higher in the endosperm than in the embryo. No consistent pattern of labelled and non-labelled cells could be clearly determined in the endosperm. The results would be consistent with a low, but variable, level of legumin occurrence in all the endosperm cells, with only the heaviest labelling being distinguished from the background. Plate 17b shows the labelling of the endosperm of a transformed seed.

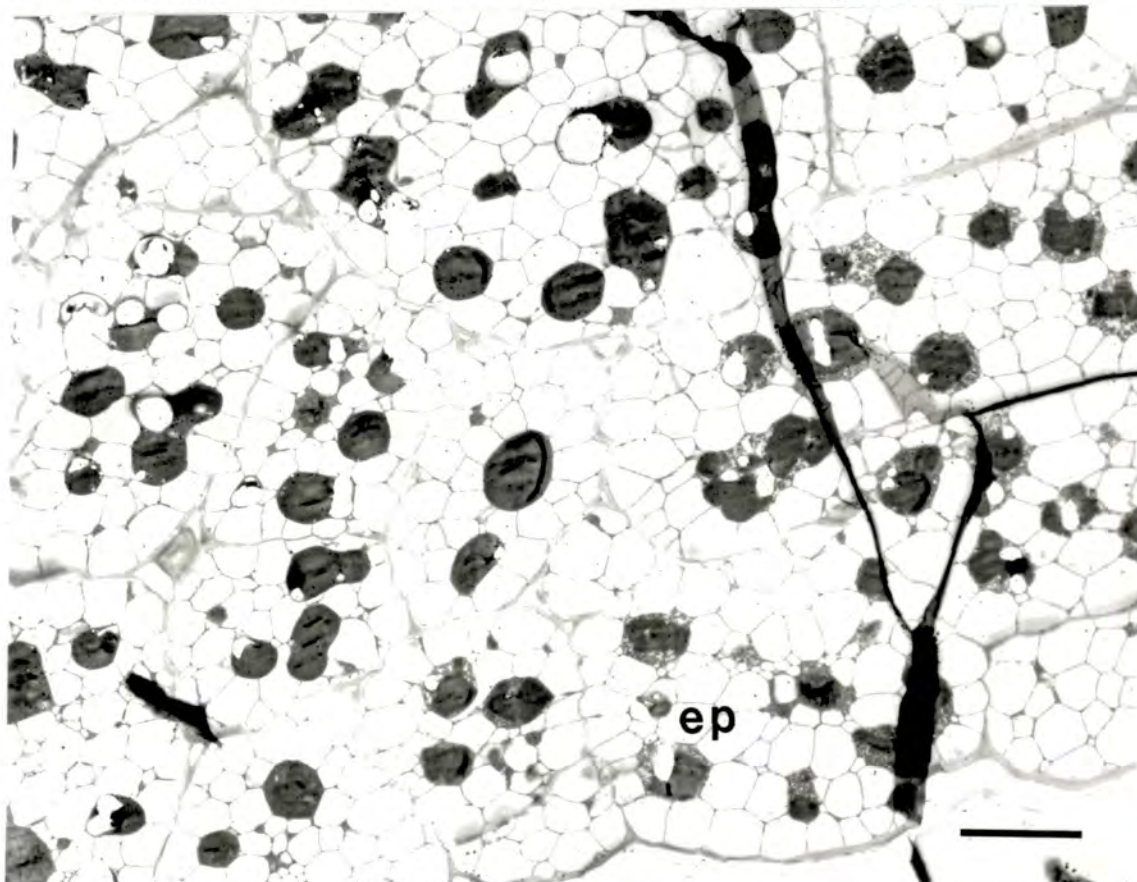
The distribution of the endogenous legumin-like protein

The labelling produced using non-pretreated antibody with sections of mature non-transformed seeds indicates the occurrence of the endogenous legumin-like protein in seeds of *Nicotiana plumbaginifolia*; Plate 13a shows the labelling in the endosperm and Plate 14a that in the embryo. There was a high intensity of labelling of all protein bodies, which was uniform in its distribution over the entire area of the protein aggregates. All endosperm cells contained labelled protein bodies and there was no apparent variation from cell to cell. In the embryo all protein bodies were immuno-labelled using the non-pretreated antisera, though the meristematic cells of the root and shoot axes did not contain any protein bodies and the proto-vascular cells had fewer protein bodies than the rest of the embryo.

Plate 16. Immunocytochemical localization of *Pisum* legumin, at EM level, in sections of IMM fixed and L.R. White embedded, *legA* transformed *Nicotiana plumbaginifolia* seed (M). Pretreated anti-legumin primary antibody, gold-labelled secondary antibody with silver enhancement, counterstained with uranyl acetate and lead citrate. Scale bar = 5 μ m.

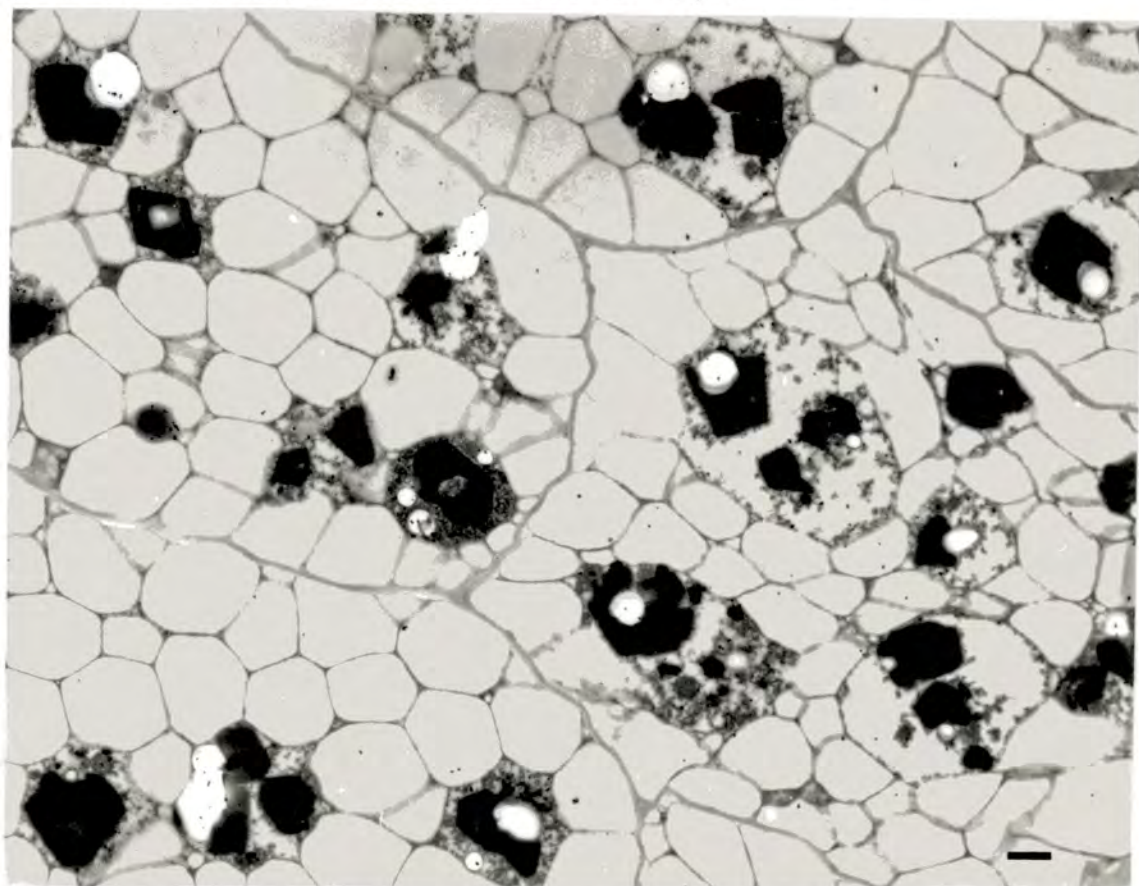


A. Labelling in the cotyledon. The protein bodies in epidermis (ep) are not labelled, cells in the core of the cotyledon contain protein bodies labelled with the characteristic peripheral pattern of labelling. There is also significant non-specific labelling of the formvar exposed by holes in the section.

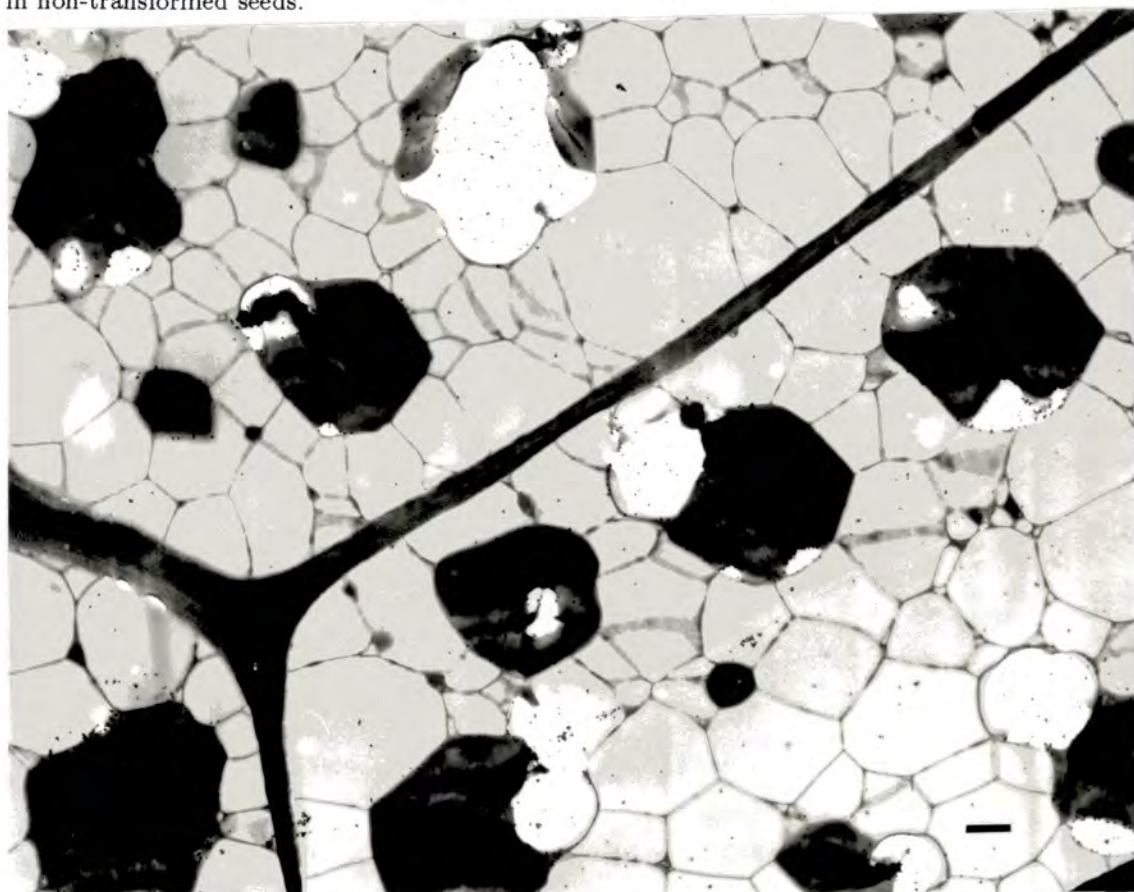


B. Labelling in the embryo axis, near the cotyledons. Epidermal (ep) cell protein bodies are not labelled.

Plate 17. Immunocytochemical localization of *Pisum* legumin, at EM level, in sections of Imm fixed and L.R. White embedded, *legA* transformed *Nicotiana plumbaginifolia* seed (M). Pretreated anti-legumin primary antibody, gold-labelled secondary antibody with silver enhancement, counterstained with uranyl acetate and lead citrate. Scale bar = 1 μ m.



A. Labelling in the radicle is at a low level, which is not significantly more intense than that observed in non-transformed seeds.



B. The intensity of labelling in the endosperm is not as great as that seen in the embryo. There is also significant non-specific labelling of the formvar exposed by holes in the section.

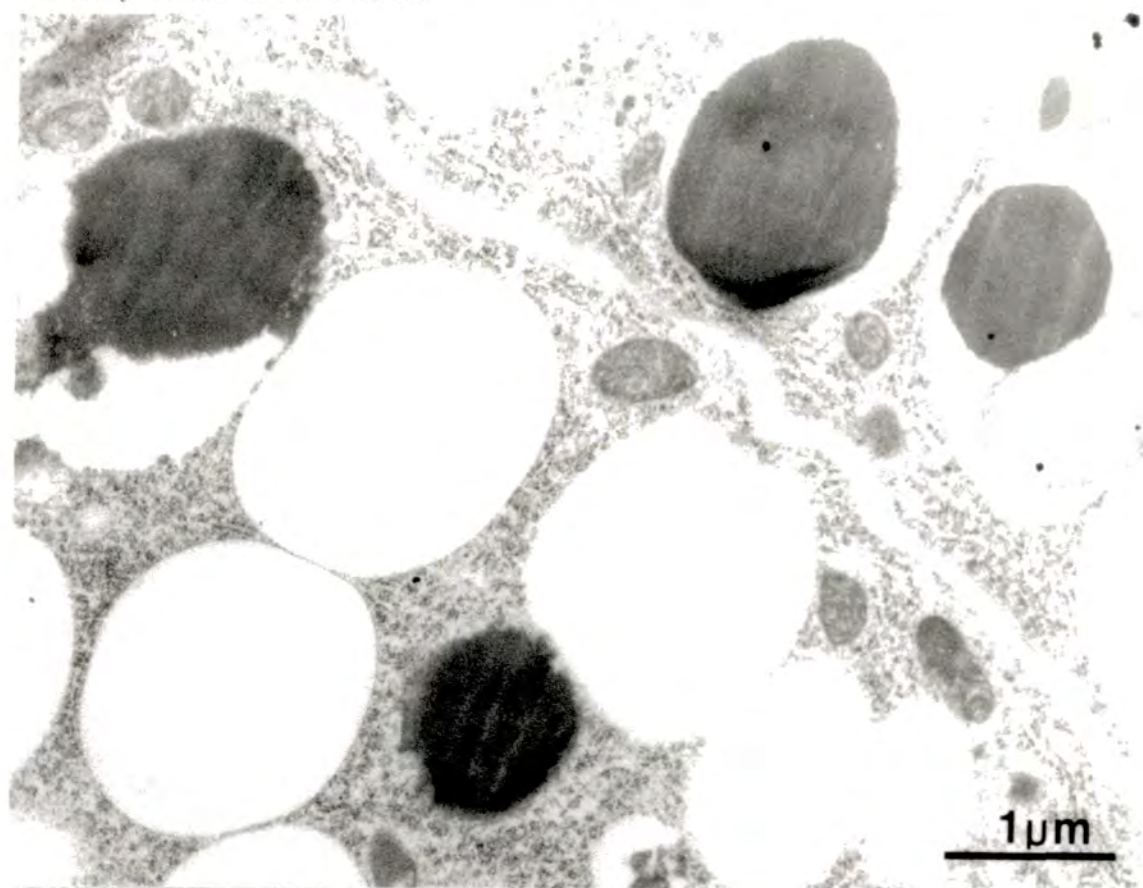
Timing of *Pisum* legumin expression

Pisum legumin was first detected by immunocytochemical localization in M-2 seeds, out of the range of developing transformed seeds that were examined. Labelling was only observed in the endosperm, as shown in Plate 18a, but there was no apparent labelling in the embryo, Plate 18b. The labelling in the seeds from the next most mature (M-1) capsules was identical in the endosperm to that observed in mature seeds, but in the embryo the immuno-labelling was of a slightly lower intensity (Plate 19) than in the mature seeds.

Timing of endogenous legumin-like protein expression

In all developing non-transformed seeds where there were any protein bodies present, these were immuno-labelled with the non-pretreated antibody. In the M-3 seeds there were no protein bodies in the embryo (Plate 20a), but in the endosperm they were present and the whole volume of the protein aggregates was labelled (Plate 20b). At the M-2 stage the seeds had protein bodies in both the endosperm and embryo and these were labelled, as shown in Plate 21.

Plate 18. Immunocytochemical localization of *Pisum* legumin, at EM level, in sections of IMM fixed and L.R. White embedded, *legA* transformed *Nicotiana plumbaginifolia* seed (M-2). Pretreated anti-legumin primary antibody, gold-labelled secondary antibody with silver enhancement, counter-stained with uranyl acetate and lead citrate.



A. In the embryo there is no *Pisum* legumin labelling.



B. The earliest observed labelling of *Pisum* legumin is in the endosperm.

Plate 19. Immunocytochemical localization of *Pisum* legumin, at EM level, in a section of an IMM fixed and L.R. White embedded, *legA* transformed *Nicotiana plumbaginifolia* seed (M-1). Pretreated anti-legumin primary antibody, gold-labelled secondary antibody with silver enhancement, counter-stained with uranyl acetate and lead citrate. Labelling in the cotyledons, but not in the epidermal (ep) cells.

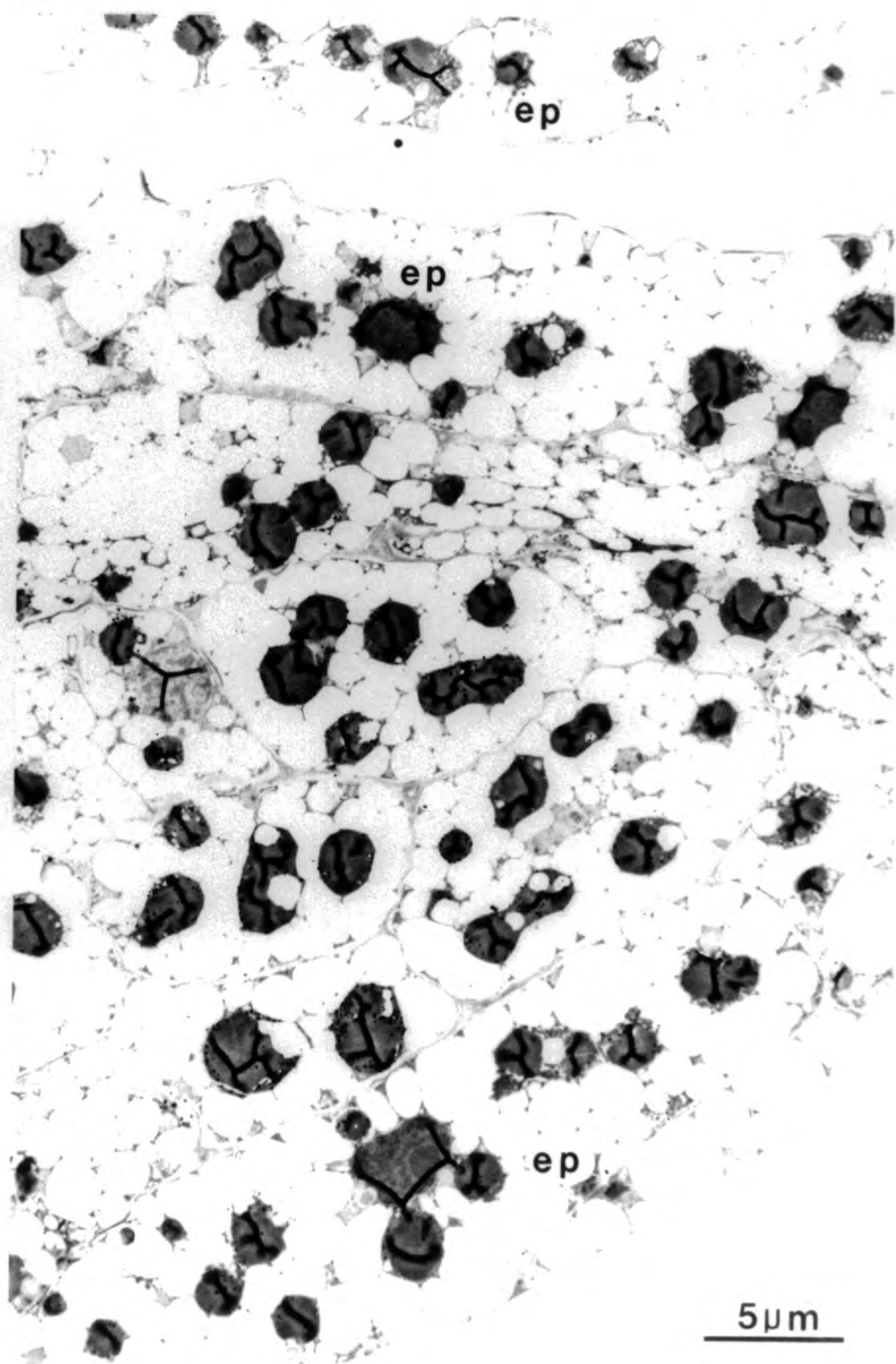
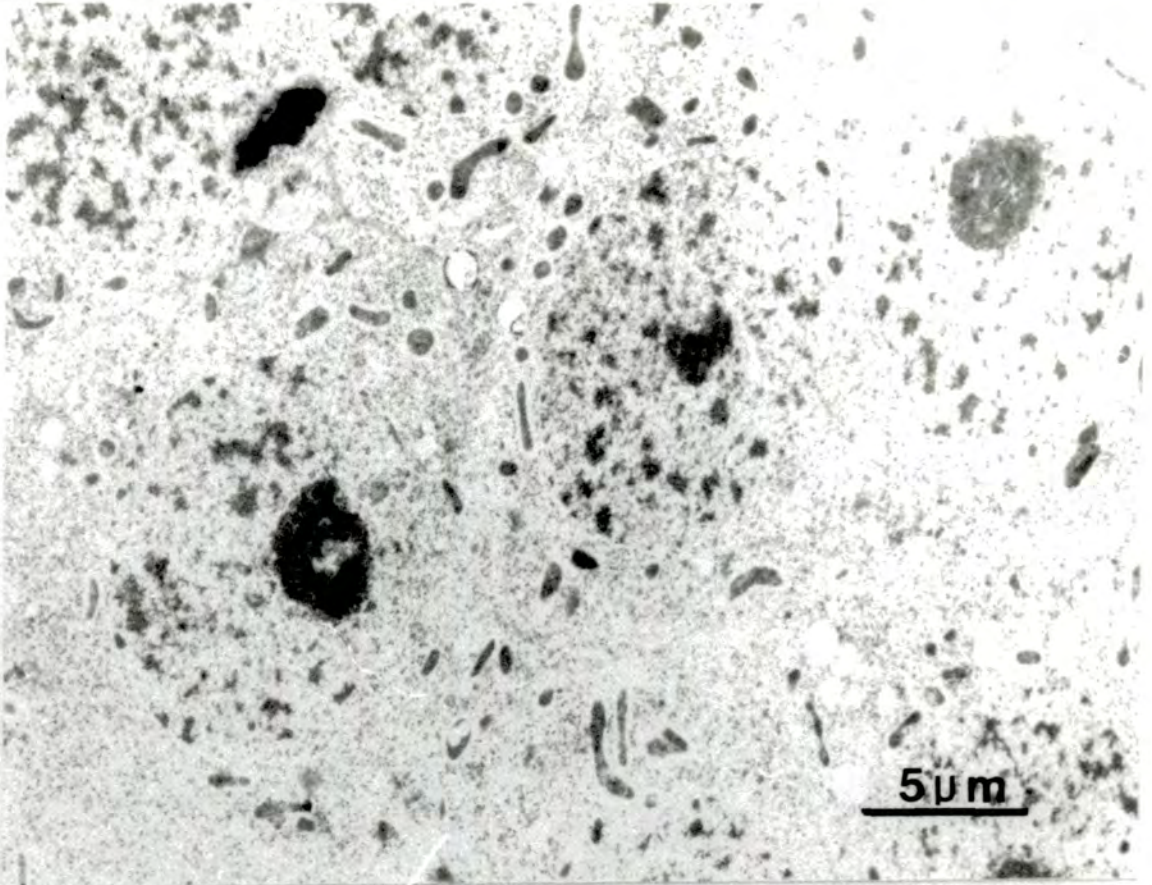
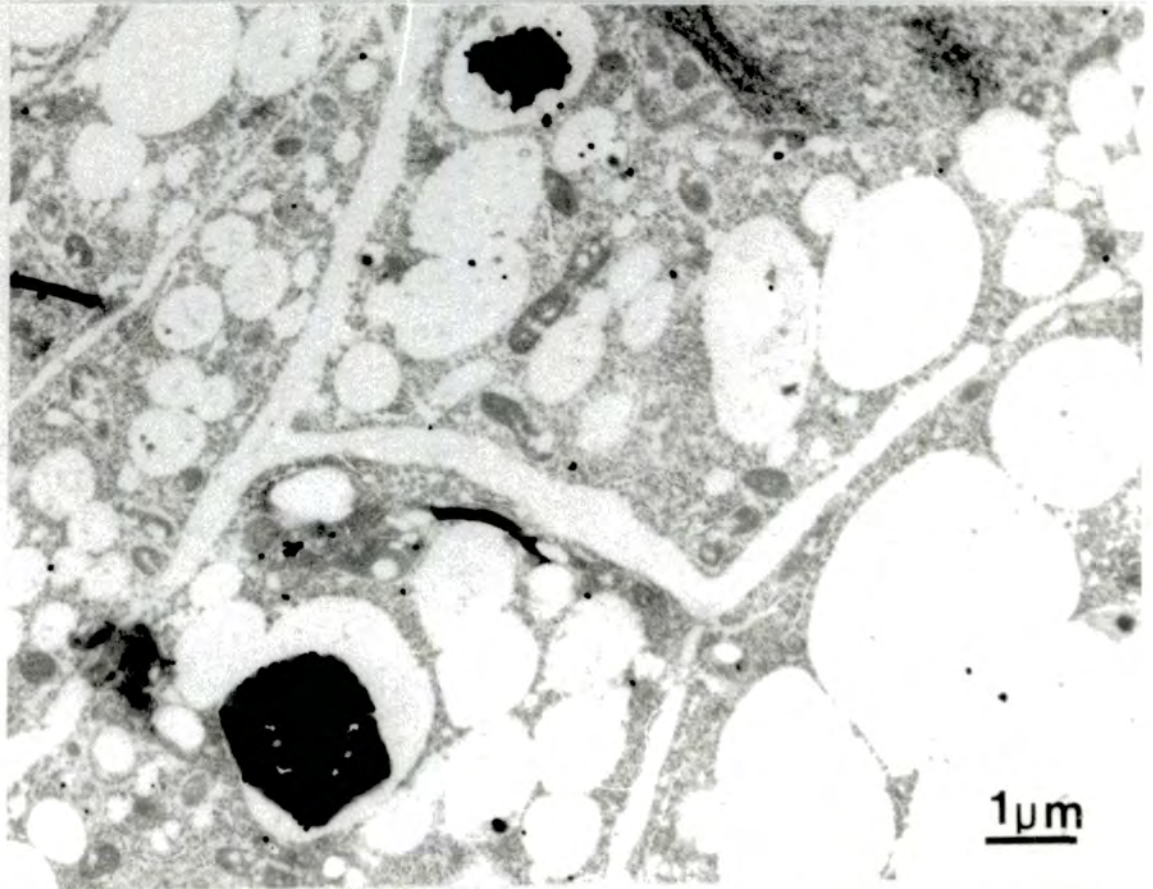


Plate 20. The endogenous legumin-like protein, at EM level, in sections of IMM fixed and L.R. White embedded, non-transformed *Nicotiana plumbaginifolia* seed (M-3).

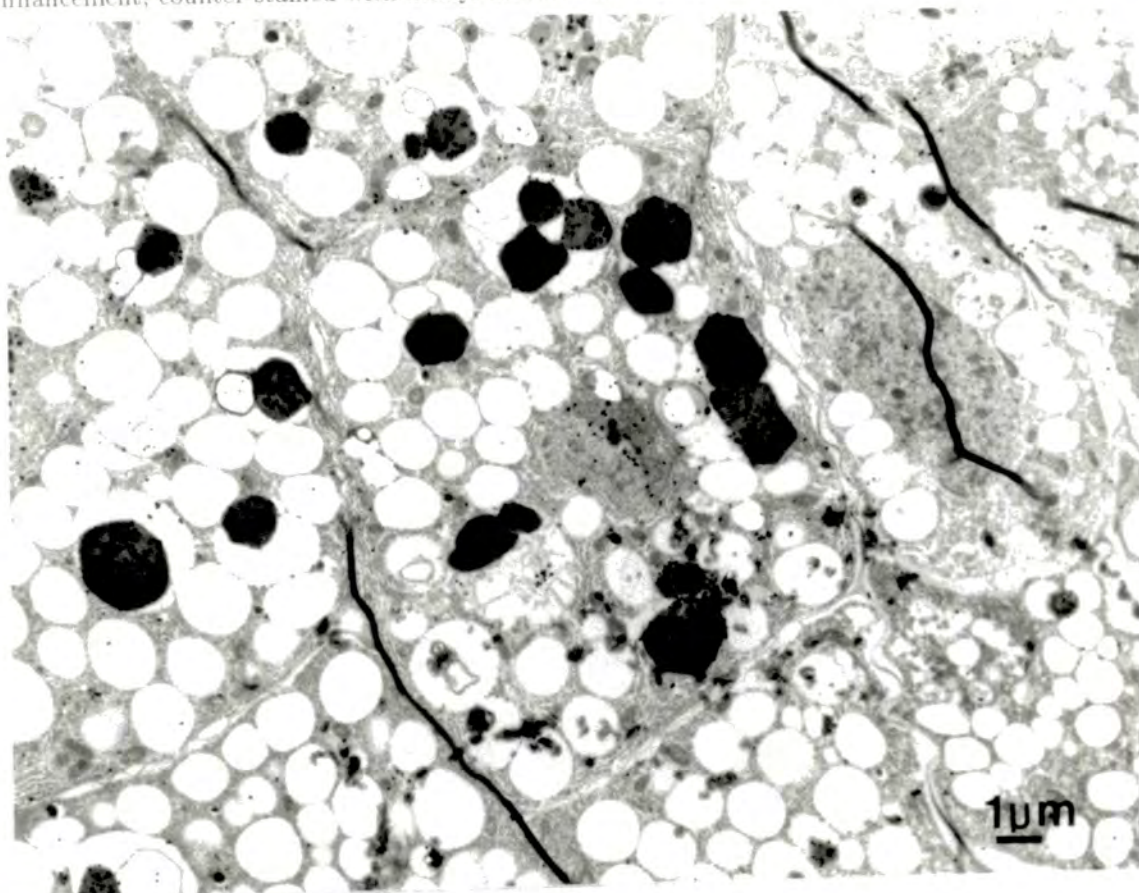


A. No protein reserves are present in the embryo at this stage of development.

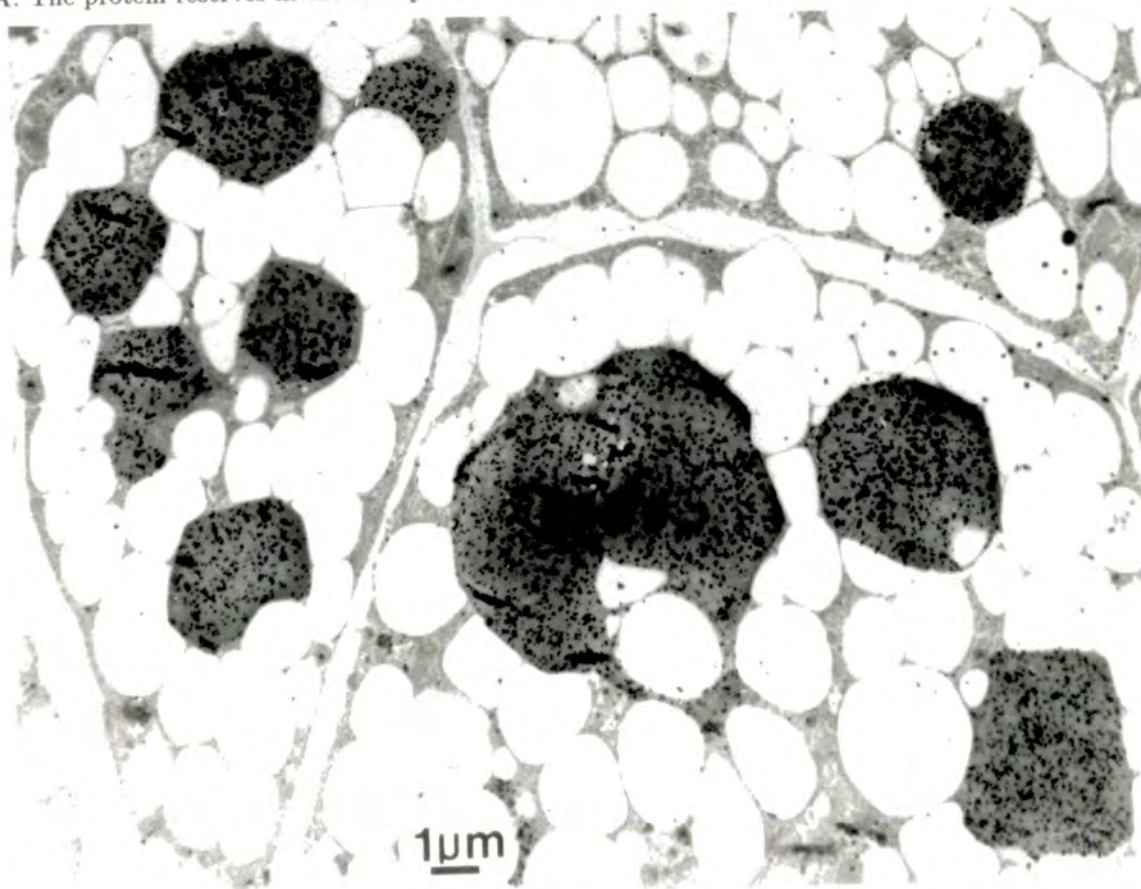


B. Immunocytochemical localization with non-pretreated anti-legumin primary antibody, gold-labelled secondary antibody with silver enhancement, counter-stained with uranyl acetate and lead citrate. The endosperm protein reserves are labelled at their earliest occurrence.

Plate 21. Immunocytochemical localization of the endogenous legumin-like protein, at EM level, in sections of IMM fixed and L.R. White embedded, non-transformed *Nicotiana plumbaginifolia* seed (M-2). Non-pretreated anti-legumin primary antibody, gold-labelled secondary antibody with silver enhancement, counter-stained with uranyl acetate and lead citrate.



A. The protein reserves in the embryo are labelled at their earliest occurrence.



B. The protein reserves in the endosperm are heavily labelled.

DISCUSSION

Selection of suitable preparation techniques

Morphology and cytology of developing *Nicotiana plumbaginifolia* seeds

The procedures used to prepare the seeds for sectioning and examination at both EM and LM level gave a good and reproducible preservation of morphology and ultrastructure. The observations made of the developing seeds were in agreement with the general descriptions of seed development, reviewed by Dure (1975) and Bewley and Black (1985), and were consistent with the more specific histological and cytological descriptions of *Nicotiana plumbaginifolia* and related species, Souèges (1920b, 1922), Avery (1933), Bhaduri (1936), Johansen (1950), Jos and Sing (1968). At the ultrastructural level, the observations made of the mature seeds were similar to the description of tobacco seed (Greenwood and Chrispeels, 1985b).

The cytological and ultrastructural investigation of *Nicotiana plumbaginifolia* seed development, especially protein deposition, was a necessary prerequisite to provide the background against which to investigate the expression of the *legA* gene in the transgenic seeds.

Immunocytochemical localization

Preparation of tissue- A standard (ST) fixation, followed by osmium tetroxide post-fixation and embedding in Spurr resin was used to produce a good, conventional preservation of ultrastructure for the investigation of *Nicotiana plumbaginifolia* seed development. This procedure would not, however, be the most suitable one to use in the preparation of tissue for immunocytochemical localization because of its adverse effects on the antigenicity of the proteins (Craig and Goodchild, 1982). For immunocytochemical localization a less rigorous fixation, IMM,

and embedding in L.R. White resin was used, which caused less damage to the antigenicity of the proteins, but this was to some extent at the expense of producing a less conventional and good preservation of ultrastructural details. In IMM fixed seeds, vacuoles and lipid bodies had the same appearance, both being un-stained. This initial ambiguity was resolved by comparisons between IMM and ST fixed seeds; in the ST fixed seeds the lipid bodies were lightly stained, whilst the vacuoles were un-stained. However, in the youngest ST fixed seeds, the lipid was more intensely stained and its appearance was similar to that of protein bodies, but these could be distinguished by similar comparisons. Such comparisons also confirmed that IMM fixation did achieve an acceptable preservation of the ultrastructural details. Similar preparation techniques, prior to immunocytochemical localization, have given acceptable results in other situations (eg. Craig and Miller, 1984; Harris and Croy, 1985) and this subject has been discussed more extensively by Herman (1988). Cryosectioning techniques would not have been applicable in this situation because of the high lipid content of the seeds, which imparts poor sectioning properties to the frozen tissues (Greenwood and Chrispeels, 1985b).

Seeds embedded in a block of resin could be sectioned conveniently with the ultramicrotome at a range of thicknesses, allowing adjacent sections of the same seed to be compared at EM and LM levels. Seeds embedded in blocks of either PEG or wax, however, could only be sectioned reliably with a microtome at thicknesses suitable for LM examination. As immunocytochemical localization was to be performed at both LM and EM levels and comparison of immuno-labelling of the same seed at both levels was desirable, L.R. White embedding was used in preference to PEG or wax.

Assessment of a range of immuno-labelling techniques- IMM fixed seeds, embedded in L.R. White resin, were used to assess post-embedding immunocy-

tochemical localization at both LM and EM levels. From the results, described above, it is apparent that the immuno-labelling in *Nicotiana* seeds could be assessed more accurately at the EM rather than LM level, because the greater resolution made it possible to identify and discount labelling caused by physical disruption of the sections or any non-specific staining. The use of silver enhancement at the EM level made it possible to use effective counter staining and examine the sections at lower powers than would otherwise be possible, so that the distribution of labelling at the tissue level could be assessed as well as at the cellular and intra-cellular levels.

Fixation and embedding have a significant effect on the physical and chemical properties of a tissue, but comparison of single seed ELISAs (Kang, personal communication) with immunocytochemical localization, demonstrated that the results achieved in both experimental formats were in general agreement. From this it was concluded that IMM fixation and L.R. White embedding does not cause any gross change to the antigenicity in *Nicotiana* seeds.

The use of pre-embedding immunocytochemical localization is described and discussed in detail by Priestly (1984). A pre-embedding procedure may reduce the extent of the damage caused to antigenic sites in the tissue, prior to incubation with the antiserum and it may avoid the obstruction of the antigenic sites, both of which can occur in post-embedding procedures as a result of dehydration and embedding. A pre-embedding approach is therefore considered to be more appropriate for some situations, for example with an antigen which is scarce or especially susceptible to damage or obstruction. However, there is only limited mobility of reagents through the thickness of non-embedded material and thus only the material close to the surface is likely to be significantly labelled, and this is especially true of plant material because of the cell walls.

The pre-embedding localization methods for the cut surface of seeds produced labelling which was hard to distinguish under the poor conditions of visibility that

could be achieved at the LM level, prior to embedding and sectioning. Hence it was impractical, in this situation, to develop this into a rapid and detailed immunocytochemical means of screening the seeds. Embedding and sectioning the seeds after immuno-labelling greatly increased the length of time required to perform immunocytochemical localization as compared with a post-embedding approach. It was also hard to find a suitable orientation of the block of tissue to cut sections through the narrow region where immuno-labelling occurred, and this was especially the case at the EM level.

Development of techniques for immuno-labelling of *Pisum* legumin

A range of immunocytochemical localization techniques were assessed for their suitability in the examination of *legA* gene expression in transgenic *Nicotiana plumbaginifolia*. A major problem, which had to be overcome, was the immuno-labelling of endogenous *Nicotiana plumbaginifolia* protein, which was apparent when non-transformed seeds, used in control experiments, were examined. A tobacco globulin, with a molecular weight of 325,000 as determined by Crowfoot and Fankuchen (1938) has been described by Derbyshire *et al* (1976) as a *Nicotiana* legumin-like protein. The immuno-labelling of protein bodies in developing, non-transformed seeds with the use of antisera raised specifically against *Pisum* legumin, would be consistent with this endogenous protein being such a *Nicotiana* legumin-like protein.

The attempts to develop a reliable immunocytochemical method, which could distinguish between the endogenous protein and *Pisum* legumin, were further complicated by the use of seeds of a mixture of genotypes produced by selfing the original transformed, hemizygous, *Nicotiana plumbaginifolia* plant. In order to ensure that both transformed and non-transformed seeds were being used in an experiment it was necessary to use as large as possible a sample of seeds. This was most readily achieved at the LM level where up to 9 seeds were cut

in one section, whereas at the EM level a much smaller area could be sectioned, allowing a maximum of 2 seeds in one section. Thus in order to take advantage of the greater resolution of the EM it was necessary to cut sections of many seeds, separately, to achieve as good a sample size. This difficulty was overcome by the identification of a homozygous legumin positive *Nicotiana plumbaginifolia* second generation plant, which made it possible to work with individual seeds known to be expressing *Pisum* legumin, and thus avoided the need for a large sample size.

The development of techniques to produce specific immuno-labelling of *Pisum* legumin was performed at the EM level, in preference to the LM level, because its greater resolution allowed smaller improvements in the quality of the labelling to be detected than would have been visible at the LM level. Enhancing a low level of labelling to make it visible at the LM level also enhances the background of non-specific labelling whilst the differential between labelling and background remains the same. Thus at the LM level the labelling was usually either too faint to see or the background of non-specific labelling was too high for specific labelling to show up. Silver enhancement at the EM level was used to investigate, at low power, the distribution of labelling at the tissue level, whilst it was also possible to check that the labelling was valid by examining the same sections at higher power.

Although changes in physical and chemical factors (eg. different buffers, washing protocols, incubation times, temperature) caused changes in the amount of labelling that was produced in immunocytochemical localization, they could not be used as a means of producing a differential in the labelling of the endogenous protein and *Pisum* legumin. The secondary antibodies and the various washing and visualization methods, which were tested in control experiments, were not responsible for any significant non-specific labelling and so they were clearly not responsible for the lack of discrimination between transformed and non-transformed seeds.

Affinity purification of the primary antibody did not produce any apparent enhancement of the differential in the immuno-labelling between the transformed and non-transformed seeds. Thus the endogenous protein may share some antigenic determinants in common with *Pisum* legumin.

Affinity purification of a polyclonal antiserum probably causes the loss of the antibodies with the highest avidity for the antigen, because these tend to remain bound to the immobilised antigen during the recovery process, whilst others of lower avidity are recovered. These low avidity antibodies often recognise more commonly occurring epitopes found on many different proteins.

The use of monoclonal antibodies for immunocytochemical localization offers the possibility of achieving very specific labelling, because the antibodies in each clonal line will recognise only a single antigenic determinant. It is likely that there will be some antigenic determinants of *Pisum* legumin which are not also present in the *Nicotiana* legumin-like protein and antibodies against these ought to give a good differential in the labelling between transformed and non-transformed seeds. However the levels of labelling that can be achieved with monoclonal antibodies will usually be lower than that which would be produced with a polyclonal serum because only one of the possible antigenic determinants is recognised. In situations where the protein of interest is expressed at a low level, the labelling intensity produced with a monoclonal antibody may be below the limits of detection, whilst a polyclonal antibody might produce detectable labelling. It is also possible to reconstruct a polyclonal antibody by pooling many different monoclonal lines, and hence achieve high levels of labelling whilst maintaining good specificity.

A range of monoclonal antibodies were assessed for their ability to discriminate between *Pisum* legumin and the endogenous protein, but it was not possible to identify any monoclonal line which produced a detectable level of immuno-labelling of *Pisum* legumin. This was possibly because the *Pisum* legumin ex-

pression was at too low a level to give a detectable level of labelling with a monoclonal antibody. However, monoclonal antibodies have been successfully used in other plant studies, particularly in the identification, characterization and localization of membrane components (eg. Bolwell and Northcote, 1984; Brewin *et al*, 1985; Norman *et al*, 1986; Villaneuva *et al*, 1986).

Pretreatment of a polyclonal antibody with an extract from non-transformed seeds caused the precipitation of some of the antibodies which recognise the endogenous *Nicotiana* protein and hence produced a significant improvement in the differential in labelling between the transformed and non-transformed seeds. Additional pretreatments produced further improvement in this differential; it is probable that each pretreatment only removed a proportion of the cross-reacting antibodies. After many successive pretreatments further slight improvement in discrimination could still be achieved by further pretreatment, but the extent of the improvement was diminished with the number of pretreatments. A Western blot was performed to compare the pretreated and non-pretreated antibody recognition of *Pisum sativum* seed extract, non-transformed *Nicotiana plumbaginifolia* seed extract and purified *Pisum* legumin. This demonstrated that the significant improvement in the specificity of the antiserum, observed in immunocytochemical localization, was also achieved in this different experimental format, which is generally considered to be a more sensitive detection system.

In ELISA, pretreatment produced a much more marked improvement in the differential between the absorbances produced with transformed and non-transformed seed extracts (Kang, personal communication). Although there are many similarities between immunocytochemical localization and ELISA, the two experimental formats differ markedly; it is known, for example, that epitope availability and binding characteristics are sufficiently different in the two formats that a monoclonal antibody that proved to be useful in ELISA is of no use for immunocytochemical localization, and *vice versa* (Weiler, personal communi-

cation). Another major difference is that in ELISA antisera are frequently used at greater dilutions, but can generate an intense signal via enzymatic reaction.

The use of the enzyme based immunocytochemical localization technique, using peroxidase and DAB to produce a reaction product, was not successful in discriminating between endogenous protein and *Pisum* legumin with the non-pretreated antibody. This enzymatic approach was not pursued with the pre-treated antibody because of the need to compare LM and EM level labelling, which could be performed more readily with the use of the immuno-gold approach.

Specific immuno-labelling of *Pisum* legumin was achieved with a polyclonal primary antibody preparation that was pretreated nine successive times before its use in EM sections of IMM fixed seeds embedded in L.R. White resin. The secondary antibody used was adsorbed onto the surface of 5 nm colloidal gold particles, which were silver enhanced. Low power EM examination was used to determine the distribution of *Pisum* legumin at the tissue level, and higher power examination of the same sections was used to determine its distribution at the ultrastructural level, and also to verify the validity of the labelling. The intensity of labelling produced in the embryo was high enough to be clearly distinguished from the residual non-specific labelling in sections of non-transformed seeds. However the lower intensity of labelling in the endosperm, coupled with a greater residual of non-specific labelling, meant that the detection of *Pisum* legumin was often less conclusive in the endosperm than in the embryo. In order to investigate the distribution of the *Nicotiana* legumin-like protein, a similar method was applied to non-transformed seeds, using a non-pretreated antibody.

The distribution of legumin

***Pisum* legumin-** In mature *legA* transformed *Nicotiana plumbaginifolia* seed, *Pisum* legumin was present in the peripheral regions of protein bodies in the

cotyledons and the adjacent embryo axis, but was not present at a detectable level in the epidermal cells or other embryo tissues, and in the endosperm it was present at a lower concentration. *Pisum* legumin is not present at a detectable level in seeds younger than the M-2 stage of development. At the M-2 stage it is only present in the endosperm but by M-1 it is also present in the embryo. In mature and the older developing *Pisum sativum* seed, legumin is present predominantly in the cotyledons, but it is also present at a lower level in the embryo axis (Harris, personal communication), the endosperm does not persist until maturity. Legumin is not present in the younger developing seeds.

***Nicotiana* legumin-like protein-** This protein was present throughout the volume of the protein bodies, in all the seed tissues and at all stages of development where protein bodies were present. Thus it was absent from the meristematic cells with had no apparent protein reserves and it was not present in the embryo before M-2 or the endosperm before M-3, respectively, when the protein bodies first appeared.

Differential gene expression

Higher plants develop from a single celled zygote, and hence all their cells are originally identical in their genetic content. As the plant matures, cells differentiate in a variety of ways in order to form the variety of tissues and cell types. In order to achieve this differentiation the plant must be regulating the same genetic information differently in different cells, at different times. It is believed that this differential gene expression is regulated by specific DNA sequences, which may be adjacent to (ie cis) or separated from (ie trans) the coding region of the DNA (Goldberg, 1983). Some of the general principles of this differential expression have been elucidated but few specific examples have been characterized in detail.

The *legA* gene is differentially regulated both in *Pisum sativum* and in transformed *Nicotiana plumbaginifolia* in order to produce the observed temporal and spatial distributions of *Pisum* legumin. In transgenic *Nicotiana* the temporal and spatial distribution of *Pisum* legumin is different to that of the similar endogenous protein and this suggests that its pattern of distribution is determined by the introduced DNA rather than endogenous sequences. In pea the endosperm tissue does not persist into the mature seed, whilst in *Nicotiana* it does and appears to have an equivalent storage function. Thus the apparent expression of the *legA* gene at a low level in the endosperm is not such a surprising occurrence, and is similar to the observed expression of *Phaseolus* lectin in transgenic tobacco (Voelker *et al*, 1987). ELISA results (Kang, personal communication) show that *Pisum* legumin is present in similar quantities in the embryo and endosperm of mature seeds, but as the endosperm is a greater proportion of the seed, it is present at a lower concentration there.

The details of the temporal and spatial distribution of the gene product give an indication of the information encoded in the gene to determine the nature of its expression. The distribution in pea is already known but it is not possible to determine which DNA sequences are responsible. By removing the gene from its usual genetic environment and inserting it into another the effects of the control sequences immediately adjacent to the coding region have been assessed without the interference of any trans-acting *Pisum* DNA sequences.

Future applications

The success of this localization method might enable legumin to be used as a marker gene in conjunction with different controlling DNA sequences in order to determine, in detail, their function. The non-coding regions of the *legA* gene might also be useful as an 'expression cassette' with the coding region of another gene which it would be desirable to express in this manner. Deletion mutations

and site directed mutagenesis are being used with this system in order to determine the functions of various parts of the non-coding sequences of *legA* (Shirsat *et al*, in preparation).

As genetic engineering of plants becomes a more practical alternative to conventional plant breeding, there is a need for suitable means of assaying the expression of the introduced genes. Such assaying will be required to detail both specificity and the 'when and where' of transgenic expression. Thus a combination of techniques will be required with for example, Western blotting providing the evidence of specific protein production and ELISA quantitation of the timing and location of such synthesis. These techniques however can only be used, at present, to look at tissues, organs or whole organisms and so necessarily involve the pooling of results from a large number of cells. The implicit assumption is that there are similar patterns of gene expression in all or most of the cells of any sample of tissue. A much greater degree of resolution can be achieved by immunocytochemical localization; cells expressing *Pisum* legumin at high levels were identified in the core of the cotyledons adjacent to the epidermal cells where there was no detectable *Pisum* legumin expression.

The methods that have been developed and the understanding that has been achieved by their application to the study of the transgenic expression of the *legA* gene should also prove to be useful for application in other situations. The use of extracts of non-transformed, but otherwise genetically identical, seeds to precipitate any antibodies which bind to it, is potentially a very powerful means of producing highly specific polyclonal antisera for use in immuno-assays of transgenic expression. The immunocytochemical localization of transgenically expressed *Pisum* legumin has been achieved with great specificity and to a high degree of resolution and so has provided a valuable means of investigating the control of differential gene expression.

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